THE JOURNAL

OF

PHARMACOLOGY

AND

EXPERIMENTAL THERAPEUTICS

VOLUME 79
1943

BALTIMORE, MARYLAND

CONTENTS

INFLUENCE OF ADDITION OF CHLORINE TO THE SIDE CHAIN ON CERTAIN ACTIONS OF ACETYLCHOLINE

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The introduction of chlorine into the molecule of aliphatic compounds usually increases the acute toxicity and intensifies the pharmacological activity (1) For example, monochloroacetic acid compared with acetic acid is considerably more toxic and more active, and it has different pharmacologic properties (2) If one considers acetylcholine an analogue of acetic acid, then it would be logical to assume that chloroacetylcholine would be more toxic and more active than the parent compound. Thus, however, was shown by Hunt and Renshaw (3) not to be the case. Comparing these two compounds they found chloroacetylcholine bromide to be less toxic and to have a muscarinic action only one thousandth as great as acetylcholine. The present studies confirm these findings and extend the observations to include additional comparative action [1]

METHODS. Blood pressure effects were studied in ten normal dogs anesthetized with pentobarbital sodium. Mean carotid arterial pressures were recorded by means of a damped mercury manometer. Chloroacetylcholine was compared in each animal with choline and acetylcholine, and all drug solutions were administered both subcutaneously and intravenously. Drug effects were determined first in the normal animal and then subsequent to either eserinization or atropinization.

The effects on smooth muscle were studied on isolated strips of rabbit ileum suspended in oxygenated Tyrode's solution at 37°C, the contractions being recorded on moving kymograph paper.

Toxicities were determined by intraperitoneal injection into adult white mice. The chromodacryorrhea test (4) was carried out on eserinized white rats, the drugs being injected intraperitoneally

RESULTS. *Blood pressure.* No significant alterations of blood pressure occurred following subcutaneous injections. The muscarinic action of chloroacetylcholine, as evidenced by its depressor action, is more nearly comparable with that of choline than with that of acetylcholine. The intravenous injection into normal animals of chloroacetylcholine in doses of 10^{-3} mols per kilogram of body weight reduced the mean blood pressure by an average of about 40 mm of mercury. Choline in like dose was about one third to one half as effective, whereas acetylcholine in equimolar solution proved fatal and was not repeated. Injection of the latter in a concentration of 10^{-6} mols per kilogram of body weight led to a depressor effect about equal to that of the choline solution (The effects of the chloroacetylcholine were somewhat more persistent). After eserinization the same dose of chloroacetylcholine produced a fall of blood pres

1

sure on an average of about 80 mm. mercury, while acetylcholine (10^{-6} mols. per kilogram of body weight) had an even greater depressor effect. The choline response was about the same as before eserinization (see fig. 1).

Nicotinic actions were demonstrated after atropinization. Chloroacetylcholine (10^{-3} mols. per kilogram of body weight) had only a slight pressor action resulting in an elevation of the blood pressure on the average of about 15 mm. mercury, whereas a like dose of choline was about twice as effective. Acetylcholine produced no rise of blood pressure in any of this group of animals until a concentration of 10^{-3} mols. per kilogram of body weight was reached, and an

Before   Atropine

After   Atropine
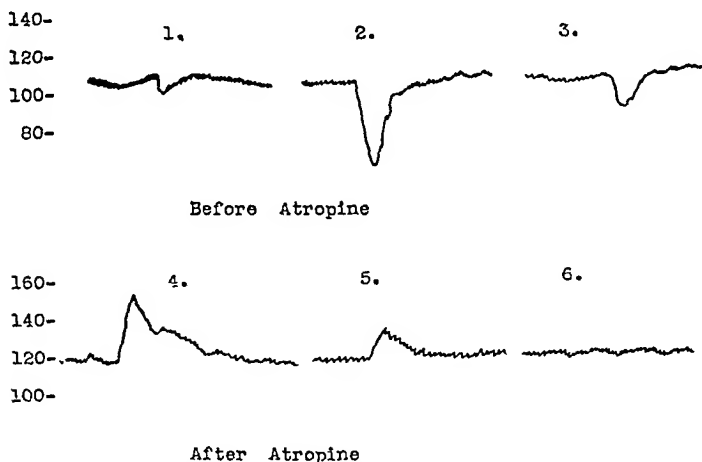
FIG. 1. Effect of intravenous injection of choline, acetylcholine and chloroacetylcholine on carotid arterial pressure of dog before and after injection of 0.05 mg./kgm. physostigmine salicylate. 1) Choline chloride, 10^{-3} Mols/kgm. 2) Chloroacetylcholine chloride, 10^{-3} Mols/kgm. 3) Acetylcholine chloride, 10^{-6} Mols/kgm. 4) Choline chloride, 10^{-3} Mols/kgm. 5) Chloroacetylcholine chloride, 10^{-3} Mols/kgm. 6) Acetylcholine chloride, 10^{-6} Mols/kgm.

increase in the dose to 10^{-2} mols. per kilogram had only a slightly greater effect (see fig. 2).

Smooth muscle. Comparable increases in tone and contractions of the ileum strips were produced by 10^{-4} molar solutions of chloroacetylcholine and 10^{-6} molar solution of acetylcholine. Choline on 10^{-4} molar solution produced only a slight increase in tone and contractions. Chloroacetylcholine as 10^{-2} molar solution and acetylcholine as 10^{-6} molar solution abolished the decrease in tone and relaxation brought about by 10^{-6} molar solution of epinephrine. However, chloroacetylcholine in concentration up to 10^{-2} molar did not antagonize the relaxing effect of 10^{-6} molar solution of atropine, while in this respect a 10^{-6} molar solution of acetylcholine produced some reversal.

Toxicity. The results are shown in table 1, and they indicate an LD 50 of 380 mgm. or 1.76 mM per kilogram of body weight for chloroacetylcholine and 200

mgm. or 1.10 mM per kilogram of body weight for acetylcholine. The results for acetylcholine are in fair agreement with those of Hunt and Taveau (5), but the results for chloroacetylcholine are higher than those obtained by Hunt and

Before Physostigmine

After Physostigmine
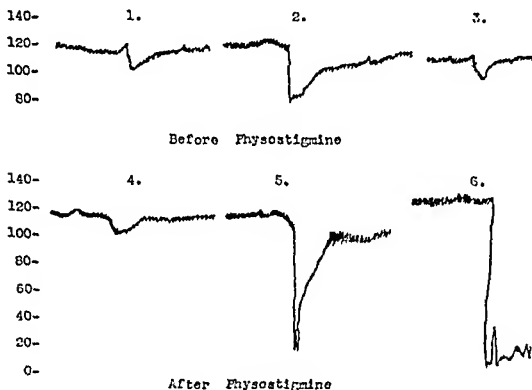
Fig. ? ...
on carc ...
sulfate ...
kgm. ...
Chloro ...

TABLE 1

Comparative intraperitoneal toxicity of chloroacetylcholine and acetylcholine for mice

	CHLOROACETYLCHOLINE				ACETYLCHOLINE		
mM/kgm.	mgm /kgm.	Lived	Died	mM/kgm.	mgm. /kgm.	Lived	Died
1.34	295	5	0	0.55	100	5	0
1 47	320	5	0	0.66	120	5	0
1.57	340	4	1	0 82	150	4	1
1 67	360	10	5	0.98	180	3	2
1.76	380	6	9	1 10	200	8	8
1.83	400	1	9	1.21	220	2	5
2 31	500	0	5	1 38	250	0	5
				1.65	300	0	5

Renshaw (4). This discrepancy may be due to different criteria being used to determine the lethal dose. Chloroacetylcholine caused more marked salivation and more prolonged and severe convulsions preceding death than did acetylcholine.

Chromodacryorrhea test.   Chloroacetylcholine in concentration up to 200 mgm. per kilogram of body weight in five different rats failed to produce "bloody tears," whereas the injection of 50 gamma of acetylcholine resulted in appearance of "bloody tears" in an average of twenty seconds in all the animals.

DISCUSSION.   Chloroacetylcholine has much weaker muscarinic action than acetylcholine, but its actions are intensified by previous eserinization, indicating that it is acted upon and hydrolyzed by choline esterase.   The duration of the depressor effects on blood vessels, however, is somewhat greater than that of acetylcholine and this would seem to indicate that the hydrolysis occurs more slowly.

Chloroacetylcholine has nicotinic action about equal to acetylcholine (pressor effect with 10^{-3} mols./kg. of both drugs), in atropinized dogs without eserine.

None of the three compounds had any significant effect on respiration.   Inclusion of chlorine in the acetylcholine molecule markedly effects its antagonistic action toward atropine as shown by the gut experiments, a relatively high concentration of chloroacetylcholine (10^{-2} Molal) having no effect on strips of atropinized gut which were affected by a very low concentration of acetylcholine (10^{-6} Molal).

SUMMARY

Introduction of chlorine into the acetyl group of acetylcholine greatly reduces its muscarinic effect on blood pressure and slightly reduces its toxicity for white mice.   Chloroacetylcholine chloride does not give a positive chromodacryorrhea test.

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FURTHER STUDIES OF THE ADDICTION LIABILITY OF DEMEROL[1]

(*1*-METHYL-*4*-PHENYL-PIPERIDINE-*4*-CARBOXYLIC ACID ETHYL ESTER HYDROCHLORIDE)

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In a previous study it was shown that Demerol, administered practically *ad libitum* to post-addicts for ten weeks, caused the development of strong physical dependence similar to that resulting from abuse of morphine (1). Andrews reported the development of tolerance to the pain threshold raising action (2) and the untoward cortical effect (3) of Demerol in these patients. These results indicate that Demerol addiction (physical dependence) and intoxication (psychosis, and epileptiform seizures) may be anticipated should this drug be abused by addicts. The decreased availability of fashionable narcotics (opium, heroin, and morphine) might lead addicts to employ Demerol as a substitute unless appropriate safeguards are taken.

It is important also to know whether or not significant addiction is apt to result from prolonged use of Demerol as a substitute for morphine in the *bona fide* practice of medicine. Batterman has administered effective clincal doses of Demerol over prolonged periods for the relief of pain without encountering abstinence phenomena on discontinuing the drug (4). Since it is known that morphine also may be administered regularly in amounts not in excess of the therapeutic requirement over a period of several months without resulting in significant physical dependence (5), Batterman's results might indicate that he used good clinical judgement as regards dosage, that Demerol is less liable than morphine to cause physical dependence in clinical use, or both.

In attempting to assay the comparative addiction liabilities of two or more drugs given for similar clinical analgesia, one must first take into account the relation of total analgetic to total physical dependence action of each drug (6). The total effect of either action can be estimated from knowledge of the intensity and duration of the effect. It might be anticipated that a drug having longer and stronger physical dependence action than analgetic effect would be more apt to cause physical dependence from repeated clinical use than a drug in which the relation of these actions is the converse, since in the latter contingency with the drug being administered on the basis of its analgetic action there would be less opportunity for sustained and cumulative physical dependence action. Such considerations pertain to the relative likelihood of physical dependence developing as a by-effect of *bona fide* clinical use, rather than to the uncontrolled abuse of a narcotic drug for the satisfaction of an inebriate impulse.

The potency of Demerol is less than that of morphine as regards both physical dependence action and analgesia. The duration of physical dependence action of Demerol probably exceeds its analgetic action only slightly and is considerably shorter than that of morphine. The duration of physical dependence action of morphine is probably at least twice that of its analgetic effect. Thus the pharmacologic characteristics of Demerol and morphine suggest that Demerol should be less apt to produce physical dependence as a by-effect of clinical use.

With these considerations in mind it was decided to ascertain whether or not the administration of Demerol in clinical doses would result in the development of physical dependence in post-addicts not requiring an analgetic. It was assumed that the clinical dose range of Demerol would be between 75 mgm. q.i.d. and 100 mgm. eight times daily.

METHODS. After at least one week of preliminary observation, Demerol was administered subcutaneously to post-addicts as shown in table 1.

TABLE 1

CASE NUMBER	DOSE OF DEMEROL	NUMBER OF DOSES PER DAY	PERIOD
	mgm.		
473, 474 and 475	100	8	2 weeks
483, 484, 485 and 487	75	4	3 months
500	75	4	2 months
500	75	8	2 weeks
515 and 516	75	8	1 month
519, 520 and 521	100	8	1 month
525 and 526	75	8	2 weeks

Cases No. 473, 474, and 475 (Subjects 1, 3, and 4 of the previous Demerol study (1)) were returned after six months of abstinence to study the effect of 100 mgm. of Demerol eight times daily for two weeks. Cases No. 515 and 516 were restudied after six weeks of abstinence as Nos. 525 and 526. The interval between the two dosage schedules shown for patient No. 500 was one day.

Observations for temperature, respiratory rate, pulse rate, and signs of withdrawal were made three times daily. Blood pressure and weight were determined each morning before breakfast. Observations were made at hourly intervals when medication was withheld for one day and for the first two days after withdrawal. Demerol was withheld for 24 hours after the first and second months of administration to patients Nos. 483, 484, 485, 487, and 500, and after the second week in patients Nos. 515, 516, 519, 520, and 521. Abstinence syndrome intensities were evaluated by the point system (1). For purposes of comparison, 5 post-addicts were given 10 mgm. of morphine q.i.d. for one month.

RESULTS. At the outset patients receiving 75 mgm. q.i.d. liked the effects of Demerol. Such effects were described as being "like Nembutal or Cocaine", and lasting only an hour or so. However, sometimes no "satisfying" effect was experienced, and during the second and third months these patients admitted of little or no "kick" from the medication. Except for occasional muscular twitching, neither the appearance nor the behavior of these patients was significantly

abnormal during the period of drug administration. In the first two weeks the patients experienced rather weired dreams, but none exhibited evidence of toxic psychosis or epileptiform seizure.

Signs of abstinence were observed upon interrupting the administration of the drug for 24 hours on the 30th and 60th days, and following its withdrawal on the 91st day. These consisted of yawning, lacrimation, rhinorrhea, perspiration, gooseflesh, and dilated pupils. No restlessness or emesis occurred. The intensity of the abstinence syndrome was at no time more than mild in any of the patients (fig. 1, curve F). The symptoms complained of were insomnia, aching
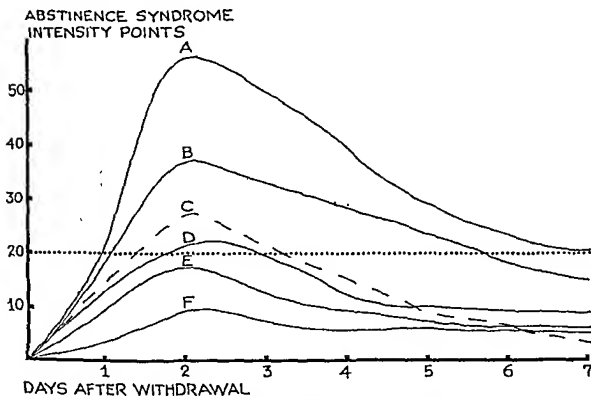
FIG. 1. COMPARISON OF ABSTINENCE SYNDROMES

B, demerol *ad libitum* for 10 weeks (1); ases #s 473, 474, 475, and 500; E, demerol, demerol, 75 mgm. q.i.d for 3 months.

in the legs, hot and cold flashes, and cramps in the stomach. None considered that he had acquired a "real habit", but rather a "mild yen" for the drug. Seventy-five mgm. of Demerol at the 30th hour after withdrawal abolished temporarily these mild signs and symptoms.

The post-addicts receiving 75 or 100 mgm. of Demerol eight times daily displayed proportionately greater effects: nervousness, irritability, and muscular twitches were noted; one had urinary retention at the outset; all slept more than usual and complained of dryness of the mouth for the first week or so. These patients also noted irregularity in subjective effects. The signs and symptoms of abstinence which followed withdrawal, while somewhat more intense than those in the group which received 75 mgm. q.i.d., would be classified as mild (fig. 1, curve E). The abstinence syndrome exhibited by patient No. 500 was of moder-

ate intensity (curve C, in fig. 1). The abstinence syndromes shown by patients 473, 474, and 475 also were of moderate intensity (curve C, fig. 1).

The changes in temperature, respiration, blood pressure, weight, and caloric intake during Demerol administration were smaller but in the same direction as those occurring in patients receiving morphine. Pulse rate tended to increase during Demerol administration, whereas it generally decreases while morphine is being given.

DISCUSSION. In interpreting the results of this study we should emphasize that the subjective estimates were made by post-addicts experienced in the effects of narcotics, that there was no clinical indication for Demerol, and that the observations for objective abstinence phenomena were made by trained observers who had no duties other than the ward administration of studies on not more than twelve patients. Two of these conditions might facilitate the development of physical dependence:

1. Post-addicts might be more susceptible to the development of physical dependence on a drug than are "normal" persons, and

2. There is reason to believe that pain antidotes effects of morphine, and perhaps Demerol.[2]

If patients receiving Demerol or morphine under clinical conditions should develop mild physical dependence, this might not be observed by hospital personnel not especially familiar with manifestations of abstinence, nor be recognized by otherwise ill and addiction-inexperienced patients. It is probable, however, that moderate or more intense abstinence phenomena would not be overlooked subsequent to discontinuation of medication.

Since such differences are in degree, not kind, it seems desirable for purposes of discussion to indicate a level of abstinence syndrome intensity which would serve to divide this illness into that which is clinically significant and that which is not. Experience suggests that abstinence syndromes reaching an intensity of 20 points or more generally are sufficiently incapacitating to warrant some therapy, whereas those below this level require little or no treatment. The latter usually are no more disturbing than a common cold or a mild attack of influenza. The abstinence syndromes of patients who received 75 mgm. of Demerol q.i.d. for three months and those given 75 or 100 mgm. eight times daily for two to four weeks fell into this latter category. Clinically significant abstinence syndromes were exhibited by the patient who was given 75 mgm. q.i.d. for two months prior to 75 mgm. eight times daily for two weeks, and by the patients previously strongly dependent on Demerol, who six months subsequently received 100 mgm. eight times daily for two weeks. Thus it would appear that previous use of Demerol, remote or recent, in large or small amounts, may facilitate the development of clinically significant physical dependence on 600 to 800 mgm. of Demerol per day. On the other hand, in two instances, 600 mgm. per day for two weeks to post-addicts with previous Demerol experience did not result in the development of clinically significant physical dependence.

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[2] Hecht, Noth, and Yonkman (8) have recently reported the appearance of abstinence phenomena subsequent to withdrawal of Demerol after its prolonged administration for the control of pain in patients not previously addicted to drugs.

The addiction characteristics of Demerol suggest that it possesses a lower order of addiction liability than morphine. The results of this study confirm this. It is not yet possible to estimate the extent of the difference in addiction liability of Demerol and morphine given in equally effective clinical doses for the prolonged control of pain, but it seems that Demerol would be the safer of the two drugs.

Since it appears probable that Demerol will soon enjoy wide therapeutic use, it is appropriate to reiterate that so far as addiction liability is concerned the difference between Demerol and morphine is largely in degree, not kind. It might be most unfortunate if the impression became popular that Demerol is "non-habit forming". Similar erroneous impressions surrounded the introduction of heroin and dilaudid and resulted in unjustified optimism and addiction in patients who should have been spared both. An analogous situation developed in certain Provinces of Canada before the sale of codeine was restricted. Hence, physicians should be enjoined to employ demerol with the same degree of caution that should be given to prescribing morphine. With such caution the beneficial effects of the drug would not be minimized appreciably and the occurrence of addiction to Demerol in the bona-fide practice of medicine should be rare.

CONCLUSIONS

1. Demerol possesses the liability of producing physical dependence similar to that caused by morphine.

2. In clinical doses the addiction liability of Demerol is less than that of morphine.

3. As an addiction preventive measure, caution and restrictions similar to those involved in the clinical use of morphine should be applied to Demerol.

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A METHOD FOR THE DETERMINATION OF DEMEROL IN URINE AND RESULTS OF ITS APPLICATION

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Demerol (hydrochloride of *1*-methyl-*4*-phenyl-piperidine-*4*-carboxylic acid ethyl ester)[1] is a synthetic drug which has certain morphine-like properties (1) (2). It is a good spasmolytic and analgetic agent and in the absence of pain will produce physical dependence in post addicts (3) if large doses are administered frequently. In addition to certain pharmacological similarities, both compounds possess a methyl piperidine group which suggested the comparison of the urinary excretion. The urinary excretion of Demerol was followed during studies on its addiction liability (4).

Barlow and Climenko (5) proposed a method for the determination of Demerol in urine using the reaction of Demerol with bromthymol blue (dibromothymol sulfonphthalein) in a buffered solution of pH 7.4 to give a yellow colored benzene soluble dye. It was found that their method was not entirely satisfactory when applied to urine having varying pH values.

Lehman and Aitken (6) have made improvements on the original technique and presented excretion data after the administration of single doses of Demerol. After the formation of the benzene soluble dye by the reaction of Demerol with bromthymol blue in buffered solution of pH 7.5, they treat it with alkali, and determine colorimetrically the amount of water soluble sodium salt of bromthymol blue liberated Their method gives satisfactory results but requires considerable time for a determination and involves large quantities of materials. The method to be described is much simpler, requires less manipulations for a determination, and utilizes much smaller amounts of bromthymol blue and benzene.

Procedure. Urine must be fresh or preserved with mercuric chloride (0.5 gm. per 24 hour sample) and kept cold. The pH of the mixture of urine and phosphate buffer[2] must be adjusted to approximately 7.5 by the addition of sufficient amounts of alkali. To determine the amount of alkali to be used 5 cc. of urine (or an aliquot portion diluted with water to 5 cc.) is placed in a test tube with 5 cc. phosphate buffer (pH 7.5) and 2 drops of stock bromthymol blue solution. The color of the resulting solution should be greenish blue to blue. If it is yellow or yellowish green, 0.5 N NaOH is added from a pipette until the desired color is obtained.

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[1] This compound, known in Europe as Dolantin, was supplied through the courtesy of Doctor O. W. Barlow of the Winthrop Chemical Company.

[2] Reagents: Phosphate buffer solution: 38.2 grams $Na_2HPO_4 \cdot 12H_2O$; 3.68 grams Na $H_2PO_4 \cdot H_2O$. The salts were dissolved in one liter of freshly distilled water.

Bromthymol blue solution (stock): 400 mgm. bromthymol blue (dibromothymolsulfonphthalein); 6.4 cc. 0.1 N NaOH; water to 100 cc. For use 8 cc. of stock solution and 30 cc. buffer solution are diluted with water to 100 cc. volume.

After determining the amount of alkali needed for the preliminary test, the same amounts of urine, water, phosphate buffer, and alkali (but no indicator) followed by 20 cc of benzene[2] are placed in a 50 cc separatory funnel. This mixture is agitated gently for three minutes and then allowed to stand until the benzene separates from the aqueous layer. Then 10 cc of the benzene extract is decanted into a 10 cc graduate cylinder and transferred to a 25 cc separatory funnel containing 5 cc of the buffered bromthymol blue solution. This mixture is also agitated for three minutes and then allowed to stand until the benzene separates from the blue aqueous solution, which is drained off and discarded. The benzene solution, which is light yellow in color, is poured from the top of the funnel into a special test tube used for reading in the Evelyn photoelectric colorimeter. After standing several
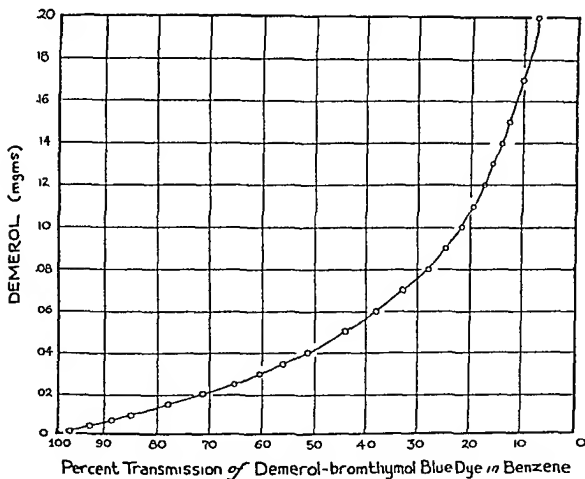
GRAPH 1

hours or over night in the stoppered tubes for clearing of any moisture held in suspension, the color intensity is read in the colorimeter, using a filter having a mean wavelength of 420 millimicrons of the transmitted band. The center setting for the apparatus is obtained by adjusting the galvanometer to 100 with a benzene extract made of the reagents in the absence of Demerol. The galvanometer reading (per cent transmission of light through the solution) is converted into mgm Demerol by means of graph 1.

Tests were made on ten post-addicts who received from 300 to 800 mgm of Demerol each day for periods varying from 1 week to 3 months (4). Twenty four hour urine collections were made each day and were analyzed for Demerol.

A number of urine samples known to be free of Demerol were tested, using 5 cc samples

for each test. A small amount of color was obtained in the final benzene solution. The average value obtained from twenty-eight (24 hour) urine samples was equivalent to 2.1 mgm. of Demerol. This value for the blank was subtracted from the total amount of Demerol found for a 24 hour urine specimen.

The excretion of Demerol after single doses was studied in several post-addicts not requiring an analgetic. Twenty-four hour urine collections were made in three successive periods of 7 (8:30 a.m. to 3:30 p.m.), 6½ (3:30 p.m. to 10:00 p.m.), and 10½ (10:00 p.m. to

TABLE 1
Recovery of Demerol added to urine

AMOUNT OF DEMEROL ADDED TO URINE	GALVANOMETER READING (PER CENT TRANSMISSION)	AMOUNT RECOVERED	DIFFERENCE	
mgm. per 2½ cc. urine	*per cent*	*mgm.*	*mgm.*	*per cent*
0.000	100			
0.005	91.5	0.006	+0.001	+20
0.005	93.0	0.005	0.000	0
0.005	91.8	0.0058	+0.0008	+16
0.010	84.0	0.011	+0.001	+10
0.010	85.0	0.010	0.000	0
0.010	*84.3*	*0.0111*	*+0.0011*	*+11*
0.010	86.0	0.0095	−0.0005	−5
0.010	84.8	0.0104	+0.0004	+4
0.0125	81.0	0.013	+0.0005	+4
0.0125	81.3	0.0128	+0.0003	+2
0.020	72.0	0.0198	−0.0002	−1
0.020	72.5	0.0192	−0.0008	−4
0.025	65.8	0.0245	−0.0005	−2
0.025	64.8	0.0256	+0.0006	+2
0.025	65 5	0.025	0.000	0
0.025	66.0	0.0245	−0.0005	−2
0.050	43.0	0.052	+0.002	+4
0.050	42.5	0.0525	+0.0025	+5
0.050	43 3	0.0515	+0.0015	+3
0.100	20 3	0.105	+0.005	+5
0 100	21 0	0.102	+0.002	+2
0.125	15.3	0.132	+0.007	+5
0.125	15.3	0.132	+0.007	+5
0.200	7.3	0 200	0.000	0
0.200	7 3	0.200	0 000	0

‚:30 a.m.) hours, respectively, before and after the administration of 100 mgm. Demerol the usual clinical dose). The blank values for the urines determined the day prior to the administration were subtracted from the total for each respective period.

RESULTS. Graph 1 is a plot of Demerol concentration against galvanometer readings prepared from varying concentrations of aqueous solutions of Demerol, using the procedure as described for urine.

The results of tests made on urine to which Demerol had been added are shown in table 1. The amounts recovered compare rather favorably with values shown in graph 1.

The relationship of intensity of color formation to urinary pH ranging from 4 3 to 8 0 was studied   When the expected amount of Demerol in urine was low and the total amount of urine used in the test was 5 0 cc , close agreements

TABLE 2

Summary of urinary excretion of Demerol

PATIENT NUMBER	DATE	NUMBER DAYS	AVERAGE DAILY DOSAGE	AVERAGE DAILY URINE VOLUME	AVERAGE DAILY EXCRETION		
					Mgm	Per cent	Per cent range
#515	10-30 to 11-12	8	600	890	46 4	7 8	4 6-11 7
	11-14 to 11-27	13	600	942	13 5	7 3	3 7-12 7
#519	11-9 to 11-12	4	300	1,227	12 8	4 3	2 7- 5 9
	11-13 to 11-22	8	800	1,345	42 2	5 3	2 9- 7 6
	11-25 to 12-6	12	800	1,205	47 1	5 9	4 1- 9 5
#520	11-10 to 11-12	3	300	1,350	20 1	6 7	5 3- 7 5
	11-14 to 11-23	10	800	1,365	74 1	9 3	2 7-15 9
	11-25 to 12-7	13	800	1,610	46 4	5 7	2 2- 8 9
#521	11-11 to 11-12	2	300	882	26 7	8 9	5 7-12 1
	11-14 to 11-24	11	730	880	89 0	12 8	8 3-20 4
	11-26 to 12-10	13	800	1,372	108 5	13 6	9 2-21 2
#522	11-11 to 11-12	2	300	1,197	21 7	7 2	6 9- 7 6
	11-13 to 11-16	4	785	1,134	62 5	7 9	2 5-11 0
#483	7-22 to 8-7	15	300	1,277	32 0	10 6	4 2-18 5
	8-9 to 8-30	17	300	1,512	34 4	11 5	5 0-16 0
	9-9 to 10-7	9	300	1,469	34 3	11 4	9 4-13 8
#484	8-12 to 8-31	15	300	1,460	12 7	4 2	2 3- 7 0
	9-12 to 10-10	8	300	1,598	13 0	4 3	2 6- 8 6
#485	7-23 to 8-8	14	300	2,554	21 0	7 0	3 2-11 9
	8-11 to 9-4	15	300	2,576	17 6	6 0	3 8- 7 1
	9-10 to 10-9	9	300	2,142	16 9	5 6	2 5-10 4
#487	8-14 to 9-9	18	300	912	35 6	11 9	6 4-18 4
	9-13 to 10-13	9	300	1,050	42 9	14 6	7 1-18 9
#500	8-31 to 9-20	18	300	888	31 7	11 5	7 1-14 7
	10-3 to 10-23	15	300	794	37 9	12 6	9 5-17 8
	10-26 to 11-5	9	600	801	74 4	12 3	8 7-18 3

in color intensity were obtained on all samples between pH 5 0 and 7 8   When higher concentrations of Demerol were present and the amount of urine used was 2 cc diluted to 5 cc with water, all samples (pH 4 3 to 8 0) tested gave close agreement with each other, indicating that the phosphate buffer, pH 7 5, and the added alkali were sufficiently effective

Table 2 shows the results on excretion of Demerol in ten patients who received the drug in daily amounts of 300, 600, and 800 mgm. for periods of 1 week to 3 months. The percent of Demerol excreted was approximately the same for each of the dosage levels, the average value for 273 analyses being 9.1. The values ranged between 2.2 and 21.2%. In some patients the average excretion value was either higher or lower than the general average for all analyses, but for any given individual the variation was within narrow limits. The development of tolerance did not appear to alter the per cent of Demerol excreted, for the values were about the same in the first and the last weeks of the study.

The excretion of Demerol after single 100 mgm. doses is shown in table 3. The average daily amount excreted is about the same as in patients receiving daily multiple doses. About 72% of the Demerol found in urine is excreted within 7 hours after its administration. In the following 6½ hours about 14%

TABLE 3

Percentage excretion of Demerol after a 100 mgm. dose

TIME OF URINE COLLECTION	SUBJECTS (POST-ADDICTS)													AVERAGE PER CENT OF DAILY AMOUNT EXCRETED
	1	2	3	4	5	6	7	8	9	10	11	12	Av.	
8:30 a.m. to 3:30 p.m. (7 hr.)	13.2	6.7	7.4	10.6	7.7	16.6	6.8	5.7	8.0	8.9	5.1	9.7	8.9	71.8
3:30 p.m. to 10:00 p.m. (6½ hr.)	0.5	2.0	1.6	2.6	0.8	0.8	2.7	2.4	2.4	tr.	2.8	2.3	1.7	13.7
10:00 p.m. to 8:30 a.m. (10½ hr.)	2.4	3.9	1.9	1.6	0.9	1.3	4.2	0.4	tr.	tr.	3.1	1.7	1.8	14.5
8:30 a.m. to 8:30 a.m. (24 hr.)	16.1	12.6	10.9	14.8	9.4	18.7	13.7	8.5	10.4	8.9	11.0	13.7	12.4	100.0

is excreted, and in the last 10½ hours of a 24 hour sample about the same amount is present. In several individuals only traces of Demerol were found in the last period.

DISCUSSION. The described test is applicable to the ester but not to the hydrolyzed product (acid) of Demerol. An amount of the hydrolyzed product equivalent to Demerol gives a very small amount of color as compared with Demerol. To what extent hydrolysis of Demerol takes place in the human organism is not known.

When a small dose of Demerol is administered a correspondingly small amount is excreted in the urine. This amount may increase the color intensity of the test only a little more than the blank which, if not determined fairly accurately, would introduce a large error. Occasionally, a Demerol-free urine gives a high value for a blank, which, when subtracted from the corresponding total Demerol in urine after its administration, would yield a false result. When large amounts of Demerol are administered the blanks become comparatively insignificant.

A number of undetermined substances present in urine apparently account for the blanks having variable values which occasionally may be unusually high An old urine without preservative will yield a high blank Some of the organic bases, such as morphine, codeine, nicotine, and aminopyrine were found to give some color by this test The amount of color developed in urine from a patient receiving 200 mgm morphine daily is so slight as to be scarcely distinguishable from a blank by this test

In addition to the fact that Demerol has much in common with morphine, the percentage excretions of both Demerol and free morphine (7) are approximately the same The duration of Demerol action is about 6 or 7 hours, after which approximately 72% of the total amount in a 24 hour sample has been excreted Small amounts can be demonstrated in urine 2 hours after a 100 mgm dose

It has been suggested (5) that the mechanism of the reaction for the formation of the benzene soluble dye is a combination of the sodium salt of bromthymol blue with the nitrogen of the methyl piperidine portion of the molecule, which is common to both morphine and Demerol The reason for the low order of color formation with the hydrolyzed product of Demerol is as yet obscure

SUMMARY

1 A method for the determination of Demerol in urine is described

2 The per cent of Demerol excreted in the urine of post addicts not requiring an analgetic receiving 300 to 800 mgm Demerol per day varied between 2 2 and 21 2 The average for 273 analyses was 9 1% Tolerance was not a factor affecting Demerol excretion

3 The average per cent excretion of Demerol after single doses of 100 mgm was, after 24 hours, about the same as in patients receiving daily multiple doses varying from 300 to 800 mgm About 72% of the total amount of detectable Demerol in urine is excreted within 7 hours after a dose

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INCREASED BLOOD SPECIFIC GRAVITY IN ANESTHETIZED DOGS FOLLOWING PILOCARPINE INJECTIONS

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Blood concentration following injections of pilocarpine has been observed by various investigators using leucocyte counts (1, 2) hemoglobin (2, 3, 4) erythrocyte counts (3, 5) and circulating water (6) as indices. It seemed however that blood specific gravity determinations which permit rapid, frequent, and delicate observations on whole blood and serum could be used profitably to investigate some of the details responsible for the concentration phenomena.

METHODS. Forty one dogs under sodium pentobarbital anesthesia (32.5 mgm./kgm.) were used. Blood pressure was taken directly by a carotid artery manometer, blood samples were drawn from the jugular and the femoral veins, and the injections of pilocarpine were made *via* the femoral vein. Blood specific gravity was determined by the falling drop method of Barbour and Hamilton (7), but a large water bath was used to insure minimal temperature changes and specially standardized pipettes mounted in a hand dropper of the general type described by Guthrie (8) were employed to obtain the standard drops of blood. Blood hemoglobin was measured by the standard Sahli method.

As blood concentration is known to vary with several factors, chief of which are the addition of erythrocytes and the withdrawal of water, the blood specific gravity during pilocarpine action was determined following the removal of the spleen (a possible source of additional erythrocytes), and the salivary glands, kidneys, stomach and intestines (structures known to remove water from the blood).

RESULTS. The specific gravity of the blood from the external jugular veins of five dogs was determined every 10 minutes for four hours, a period exceeding the duration of any of the pilocarpine experiments. These controls showed that there was a slight gradual rise in blood specific gravity during the four-hour test (fig. 1). Consequently, the curve drawn from these averages has been placed on every graph for comparison.

The average normal specific gravity of the blood from another series of five dogs was found to be 1.0581 (table 1). Determinations were made every five minutes for 20 minutes to establish a normal value under the existing conditions of anesthesia and surgery. At this time pilocarpine nitrate was injected (0.2 mgm./kgm. same dosage throughout this work) and the blood specific gravity followed at 5 minute intervals. The highest blood specific gravity (1.0644, table 1) indicating the maximal blood concentration was attained 5 minutes after the injection. At the end of 20 minutes the average specific gravity had decreased to 1.0619, and a second injection of pilocarpine nitrate was given. Five minutes after this second dose the highest level was noted (1.0652) and fifteen minutes later the specific gravity was still 1.0636 (table 1).

The blood pressure fluctuated very little after an initial fall of about 50 mm. of mercury immediately following injection of pilocarpine. After about three

16

minutes the blood pressure returned to normal in this experiment and all others reported in this study.

In a third series of three dogs the blood specific gravity rose definitely during the first 5 minutes following pilocarpine injection (the average increase being 0.0076, figure 1). The specific gravity of the serum of the blood from these dogs was also determined. Two dogs showed a rise in specific gravity and one a fall at the end of 5 minutes after pilocarpine injection, although the blood specific
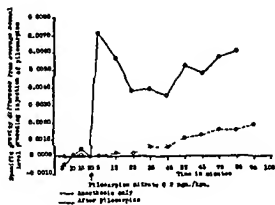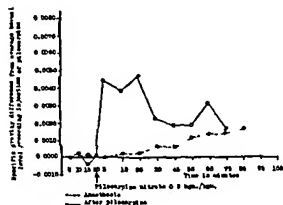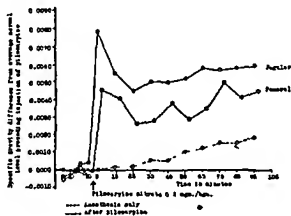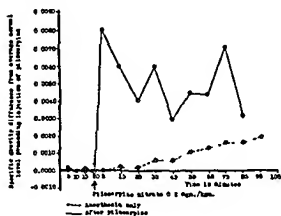
vary glands from dogs.

gravity of all 3 dogs rose during this period. Subsequently, the serum specific gravity was determined for blood drawn from 12 other dogs (modified by various surgical procedures, table 2) following pilocarpine injections. In 11 of these dogs the serum specific gravity rose and one showed a slight fall, that is from a total of 15 dogs the specific gravity of the blood serum rose in 13 and fell in two during the 5 minute period of maximal blood concentration following pilocarpine injections It seems, therefore, that either water is lost from, or there is some addition of solids to the blood serum during this period of maximal concentration.

The hemoglobin of the dogs in series 3 increased from an average normal level of 14.3 grams to 19.2 grams per 100 cc. of blood during the 5 minute period of blood concentration following pilocarpine injection (table 2).

As pilocarpine causes marked salivation a comparison of the specific gravity of blood from the jugular and femoral veins of five dogs (the femoral samples drawn two minutes after the jugular) was made to determine any changes the activity of the salivary glands might have on the concentration of the blood. The average specific gravity of the blood from these five dogs showed that the normal specific gravity of the blood from the jugular vein was very slightly higher than that of the blood from the femoral vein (fig. 1). Five minutes after the injection of pilocarpine specific gravity values of the blood from the jugular and femoral veins were at the highest levels, 1.0618 and 1.0584, as compared to the normal values of 1.0539 and 1.0537 respectively. At the end of 95 minutes the specific gravity levels of the blood from the jugular and femoral veins (1.0598

TABLE 1

Effect of two injections of pilocarpine on the specific gravity of dog blood

DOG	NORMAL*				AFTER PILOCARPINE†					AFTER SECOND DOSE OF PILOCARPINE‡				
	5 min	10 min	15 min	20 min	2 min	5 min	10 min	15 min	20 min	2 min	5 min	10 min	15 min	20 min
11	1 0646	1 0635	1 0630	1 0630	1 0637	1 0656	1 0638	1 0636	1 0628	1 0656	1 0672	1 0655	1 0551	1 0639
12	1 0596	1 0594	1 0591	1 0588	1 0654	1 0659	1 0654			1 0676	1 0679	1 0661	1 0654	1 0651
13	1 0563	1 0562	1 0567	1 0566	1 0597	1 0614	1 0603	1 0599		1 0569	1 0599	1 0617	1 0610	
14	1 0551	1 0551	1 0551	1 0551	1 0612	1 0639	1 0634	1 0636	1 0617	1 0639	1 0564	1 0661	1 0654	1 0634
15	1 0570	1 0560	1 0570	1 0569	1 0651	1 0651	1 0606	1 0621	1 0611	1 0642	1 0648	1 0634	1 0630	1 0620
Aver	1 0585	1 0580	1 0582	1 0581	1 0630	1 0644	1 0627	1 0623	1 0619	1 0636	1 0652	1 0646	1 0640	1 0636

* Time elapsed after surgery completed
† Time elapsed after injection of 0 2 mgm /kgm pilocarpine nitrate
‡ Time elapsed after injection of 0 2 mgm /kgm pilocarpine nitrate

and 1.0584) were still 0.0059 and 0 0046 higher than the normal values respectively (fig. 1).

As that difference persisted much longer than the actual increased salivation, it cannot be explained solely on the basis of increased activity of the salivary glands.

As this difference was greater during the first few minutes after pilocarpine the loss of fluid through the salivary glands might account for at least part of the initial rise in specific gravity of the blood. Therefore those glands were removed from three dogs and the determinations of specific gravity made. The average normal specific gravity level of the blood for the 20 minute period preceding injection was 1.0561 and the highest rise (which occurred in five minutes) was 0.0071 (fig. 1). At the end of 85 minutes the specific gravity level was still above normal (0.0061, fig. 1). At the five minute period the serum showed an increase from a specific gravity of 1.0256 to 1 0294 during profuse salivation of the dog, which indicated a loss of water from the plasma (table 2). That loss could account for a part of the early rise in specific gravity of the whole blood.

The stomach which usually secretes in response to pilocarpine stimulation was removed from each of three dogs and pilocarpine subsequently was injected, resulting in a maximal rise of 0 0105 in 55 minutes (fig 2) It should be stated however, that there was a previous rise of 0 0086 in 25 minutes At the end of

TABLE 2

Effect of pilocarpine on the whole blood and serum of dogs

	DOG	BLOOD SPECIFIC GRAVITY			SERUM SPECIFIC GRAVITY			HEMOGLOBIN CONTENT GM /100 CC		
		Normal*	After pilocarpine		Normal	After pilocarpine		Normal	After pilocarpine	
			5 min	85 min		5 min	85 min		5 min	85 min
No organs removed	11	1 0556	1 0640	1 0591	1 0218	1 0264	1 0256	15 0	21 0	18 5
	12	1 0551	1 0636	1 0559	1 0275	1 0310	1 0275	15 0	20 0	16 0
	13	1 0502	1 0565	1 0554	1 0205	1 0261	1 0200	13 0	17 0	14 5
Average		1 0536	1 0615	1 0568	1 0263	1 0278	1 0214	14 3	19 3	16 3
Salivary glands removed	19	1 0505	1 0563	1 0537	1 0258	1 0283	1 0249	12 2	15 4	13 6
	20	1 0638	1 0685	1 0691	1 0270	1 0291	1 0275	15 0	18 2	19 0
	21	1 0540	1 0649	1 0637	1 0240	1 0305	1 0291	14 2	18 2	17 6
Average		1 0561	1 0632	1 0622	1 0256	1 0294	1 0272	13 8	17 3	16 7
Stomach removed	22	1 0584	1 0657	1 0677‡	1 0346	1 0319‡	1 0359‡	16 0	19 0	19 2‡
	23	1 0527	1 0632	1 0521‡	1 0173	1 0179‡	1 0188‡	15 4	18 2	19 2‡
	24	1 0569	1 0600	1 0590‡	1 0207	1 0215‡	1 0229‡	16 4	17 8	18 6‡
Average		1 0560	1 0630	1 0596	1 0242	1 0264	1 0259	15 9	18 4	19 0
Kidneys removed	25	1 0407	1 0467	1 0440	1 0176	1 0200	1 0185	11 4	12 6	12 0
	26	1 0568	1 0594	1 0600	1 0258	1 0330	1 0301	13 0	15 5	15 0
	27	1 0485	1 0501	1 0514	1 0220	1 0224	1 0270	11 0	11 5	11 4
Average		1 0487	1 0521	1 0518	1 0218	1 0240	1 0252	11 8	13 1	12 8
Circulation of intestines ligated	33	1 0612	1 0654	1 0592‡	1 0270	1 0282‡	1 0261‡	16 1	19 2	16 0‡
	34	1 0584	1 0599	1 0591‡	1 0313	1 0303‡	1 0303‡	15 8	16 8	15 8‡
	35	1 0617	1 0658	1 0615‡	1 0316	1 0324‡	1 0316‡	18 0	19 5	18 0‡
Average		1 0604	1 0637	1 0602	1 0299	1 0303	1 0293	16 6	18 5	16 6

* Time elapsed after surgery completed (twenty minute average)

† Time elapsed after injection of 0 2 mgm /kgm pilocarpine nitrate

‡ 75 minutes after pilocarpine

75 minutes the specific gravity level had fallen to 1 0596 (fig 2) Again the specific gravity of the serum had increased slightly (1 0242 to 1 0264) while the hemoglobin content of the blood rose from 15 9 to 18 4 grams per 100 cc of blood in five minutes (table 2) In contrast to other experiments previously run in this study the specific gravity reached the highest peak in 55 minutes

injected. These results suggest that histamine and pilocarpine may operate *via* the same mechanisms to produce blood concentration.

DISCUSSION. The experimental results indicate that pilocarpine produces a sharp temporary rise in the specific gravity of the blood throughout the body, as well as in the blood vessels draining those organs specifically affected by pilocarpine. At least two conditions contribute to this rise in specific gravity of blood, namely an increase in the number of red blood corpuscles per unit of blood, and a loss of water from the circulating fluid. The increased number of red cells apparently results predominantly from the action of pilocarpine on the spleen and possibly other red cell reservoirs throughout the body. The loss of water from the blood under the influence of pilocarpine seems to be chiefly by way of the kidneys and intestines and to a lesser extent through the stomach, salivary glands, and the tissue spaces. The loss of fluid from the blood is suggested rather than an increase in solids in the blood as the organs removed are not believed to produce blood proteins, but (excepting the pituitary) are known to remove water from the blood during their normal activities. Furthermore, if an increase in solids had occurred, then all of the dogs should have responded similarly regardless of the organs removed.

SUMMARY

1. The specific gravity of blood of dogs lying quietly under sodium pentobarbital anesthesia (32.5 mgm./kgm.) rises gradually.

2. Pilocarpine nitrate (0.2 mgm./kgm.) when administered to dogs in deep pentobarbital anesthesia produces a rapid increase in the specific gravity of the blood, the maximal rise being five minutes after the injection of the drug.

3. The spleen, the kidneys and the intestines collectively contribute in the production of this rise in blood specific gravity following injection of pilocarpine into anesthetized dogs.

4. No evidence that the posterior pituitary gland is involved in this rise in blood specific gravity following injections of pilocarpine was obtained during these experiments.

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THE REDUCTION OF SYMMETRICAL TRINITROTOLUENE BY A SUCCINIC DEHYDROGENASE PREPARATION

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In previous work it had been observed that tissues of animals incubated *in vitro* were able to form amino compounds from trinitrotoluene (1). It had also been noted that succinate added to tissue extracts stimulated this reduction of the nitro group by these extracts. In addition it had been found that heating the extracts to 80°C almost completely destroyed their reducing activity. These findings seemed to indicate that an enzyme concerned in the activation of hydrogen might be the agent catalyzing the reaction. The effect of the succinate particularly pointed toward the involvement of succinic dehydrogenase. Since this enzyme is known to have such a prominent part in respiratory metabolism (2) it was chosen for study, both because of the above findings and because a relatively stable, active preparation was easily made (3). The studies reported here show that this crude preparation is active in the reduction.

METHODS AND MATERIALS. The succinic dehydrogenase used was prepared from frozen beef heart by an established procedure (3). While this preparation was rather crude,[1] it seemed more active than that obtained by any other method and reduced methylene blue in six minutes (3, 4, 5). For comparative purposes preparations were also made on a small scale by this procedure from rat, guinea pig and chicken hearts.

As with the tissue extracts the reduction was carried out anaerobically by the Thunberg technique. Removal of proteins and color development were carried out essentially as previously outlined (1). The 2,6-dinitro-4-amino toluene as standard for this was much more satisfactory than when used with the material resulting from the interaction of kidney or liver extracts and the substrate.

Commercial reagent grade succinate, lactate and citrate were used as their sodium salts. Sodium malonate, fumarate, pyruvate and diselenodiacetate were prepared by neutralizing the acids. The sodium selenite was reagent grade material extensively used in this laboratory for toxicity and chemical studies in recent years. Diselenodiacetic acid was also a portion of that previously used (7). The succinate and other metabolites unless otherwise stated were used at such a concentration as to be 0.01 M in the reaction tube after mixing.

In expressing results it has been assumed that the major portion of the material formed by the reducing activity of the preparation is 2,6-dinitro-4-aminotoluene or 4,6-dinitro-2-aminotoluene. If the NO₂ group of the number 6 position were reduced instead of the 4 or 2 it should give a product identical with the latter. In any event if the reduction does not go beyond the mono amino stage the synthetic 2,6-dinitro-4-aminotoluene should probably serve as a satisfactory standard. In practice this was found to be the case. The amount of reduction is recorded as mgm of 2,6-dinitro-4-aminotoluene per 100 grams of enzyme preparation. The latter had about 45 mgm protein per cc. The variables of color comparison, temperature control, concentration of solution due to evacuation and

the manipulative procedures were such that duplicate estimation differed by as much as 10 per cent. The resulting figures have accordingly been rounded off within these limits.

The compound
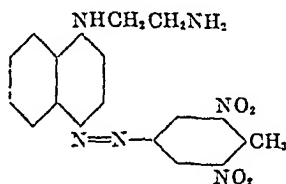
presumably formed by the diazotization and coupling reaction had a clear pink hue well suited to colorimetric comparison with the Klett colorimeter.

RESULTS. As in the work (1) with the extract of the whole tissues the optimum pH for the reduction was found to be 7.4. Table 1 shows the effect of temperature on the reduction. The enzyme preparations were maintained at

TABLE 1

Reduction of TNT as influenced by various temperatures pH 7.4 maintained with phosphate buffer

TEMPERATURE	AMINO COMPOUND FORMED PER 100 GMS. ENZYME PREPARATION
°C.	mgm.
2	2
12	14
25	57
37.5	95
43	74
55	16
80	4

the stated temperature for 30 minutes before adding the TNT and succinate and were then incubated for an hour as usual at the given temperature. It will be noted that the reaction was very nearly abolished at 2 and 80°C. If, however, these two tubes were subsequently incubated at 37.5°C. with added succinate for an hour the former gave a value close to 95 mgm. per cent, while the latter showed no increase as was to be expected if this preparation were behaving as the usual succinic dehydrogenase.

The progress of the reaction with time is illustrated by the data of table 2.

Some other possible hydrogen donators were tried with the preparation in place of the succinate. Pyruvate, citrate, fumarate, malate and glucose were without stimulating effect. The findings showed simply that the preparation was relatively pure, *i.e.*, free of such other enzymes as fumarase. Lactate produced a slight reduction, presumably due to some lactic acid dehydrogenase in the preparation.

The effects of various agents known to influence the activity of succinic dehydrogenase are set forth in table 3. The results are expressed as percent

of the reduction obtained with the standard amount (0 01 M) of added succinate   The enzyme was first incubated for an hour with the added agent, then succinate and TNT added and the incubation continued for another hour   Ascorbate and iodoacetate seemed to produce an inhibition of the reduction, but it was found that both these interfered with the diazotization procedure in the hydrochloric acid solution used for estimating the extent of the reaction

TABLE 2

Amount of reduced material formed from the TNT at stated time intervals
Temperature = 37 5°C , pH 7 4

TIME	MGM PER 100 GRAMS PREPARATION
minutes	
0	0
10	24
20	44
30	59
40	76
50	87
60	97

TABLE 3

Effect of various agents on reduction of TNT by succinic dehydrogenase in the presence of 0 01 M succinate pH 7 4

Temperature 37 5°C   Per cent reduction obtained by 0 01 M succinate + TNT + enzyme taken as 100

PREPARATION NUMBER	CONTENT OF PREPARATION	REDUCTION
		per cent
1	TNT + succinate (0 01 M)	0
2	TNT + enzyme	5
3	TNT + enzyme + succinate (0 01 M)	100
4	TNT + enzyme + succinate (0 04 M)	115
5	TNT + selenite (0 01 M) + enzyme	0
6	TNT + malonate (0 01 M) + enzyme	0
7	TNT + enzyme + pyrophosphate (0 01 M)	100
8	TNT + enzyme + diselenodiacetic acid (0 01 M)	95
9	TNT + enzyme + ferricyanide (0 01 M)	10
10	TNT + enzyme + oxygenation	0

In the case of the former, partial correction could be obtained by adding cyanide to the protein free filtrate with subsequent diazotization   This was never wholly satisfactory, however, and since the cyanide is itself a reducing agent with unascertained results it is not included in the above tabulation

Certain differences in the behavior of the inhibitors are noteworthy with respect to the time needed for inactivation of the enzyme preparation   Thus, malonate needed an hour's incubation with it previous to addition of the suc

cinate and TNT, whereas selenite gave the same end result whether incubated with the enzyme for an hour or added at the same time as the succinate and TNT.

The preparations were ordinarily used the day they were made from the frozen heart. The activity decreased slowly when the suspension was stored at 5°C. dropping to 80 per cent of the original in 10 days. The frozen heart itself continued to give potent preparations after 6 months storage in that state.

An effort was made to ascertain the effect of possible end products of the reduction such as *2,6*-dinitro-*4*-hydroxylaminotoluene or *2,6*-dinitro-*4*-aminotoluene on the course and extent of the reaction but this proved to be very difficult under the circumstances. Both of these diazotize and combine with the color reagent so that their value had to be subtracted from that given by the product formed from the TNT. Also the hydroxylamino compound seemed to undergo some change during the incubation, probably further reduction. Accordingly this attempt was abandoned.

What bearing the reduction of the nitro groups has on the toxicity of TNT to the animal organism remains to be determined.

SUMMARY

1. A succinic dehydrogenase preparation has been examined for its reducing action on symmetrical trinitrotoluene.

The preparation rapidly reduces the toluene derivative to a product which behaves on diazotization like the 4 amino compound.

2. The reduction is influenced by various agents to a similar extent and in much the same manner as has been established by others for the influence of these agents on the activity of this preparation as a dehydrogenase.

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ANTAGONISTIC EFFECT OF N-ALLYL-NORMORPHINE UPON MORPHINE

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Desmethylation of morphine, codeine and other related alkaloids does not change qualitatively the action of these alkaloids, although their analgesic effects are considerably diminished (1). The substitution of the NH group in norcodeine by a N-allyl group (2), however, produces a remarkable alteration in the pharmacologic action of this substance. Pohl (3) found that N-allyl-norcodeine stimulated the respiration and antagonized the depressing action of morphine upon the respiration. Furthermore, he described an experiment in which N-allyl-norcodeine temporarily aroused a dog from sleep induced by morphine. According to McCawley, Hart and Marsh (4) and also to Hart (5) N-allyl-normorphine appears to have a stronger antagonistic action toward the depression of the respiration evoked by morphine than N-allyl-norcodeine. Upon the suggestion of Chauncey D. Leake, N-allyl-normorphine was studied pharmacologically in comparison with morphine.

The N-allyl-normorphine used in this study was synthesized by Weijlard and Erickson (6) in the Merck Research Laboratories. Since the alkaloid is but slightly soluble in water, it was dissolved by the addition of a few drops of hydrochloric acid; the final solution (1% and 3% respectively) were adjusted to a pH of 5 or 6 by adding sodium hydroxide.

General Effects. In dogs, subcutaneous injections of 10 or 20 mgm. per kgm. of N-allyl-normorphine produced no signs of depression or analgesia; the animals remained alert and moved about freely. No change in the rate of respiration was noted. Defecation occurred occasionally within 30 minutes after the injection. Morphine (5 or 10 mgm. per kgm.) given 20 to 60 minutes after the injection of N-allyl-normorphine failed to produce vomiting, drowsiness or muscular incoordination, manifestations otherwise regularly obtained in these dogs with 5 or 10 mgm. per kgm. morphine. Apparently premedication with N-allyl-normorphine renders the subsequent injection of morphine entirely ineffective when given within an hour after the N-allyl-normorphine. The effect of morphine is markedly attenuated when it is given 2 or 3 hours after the administration of N-allyl-normorphine.

Dogs intoxicated by morphine (5 mgm. per kgm.) were promptly aroused from their drowsiness by a subsequent injection of N-allyl-normorphine. Within 15 minutes the manifestations of morphine intoxication vanished almost completely and the dogs regained their normal behavior.

Cats tolerated subcutaneous injections of 10 or 20 mgm. per kgm. of N-allyl-normorphine without showing significant changes in their behavior. Subcutaneous injections of morphine (5 mgm. per kgm.) which otherwise produced

restlessness, marked irritability, incoordination and extreme mydriasis, failed to produce any visible effects when given to the same animals 30 minutes after the administration of N-allyl-normorphine. Also, the effects of morphine were promptly abolished by N-allyl-normorphine given 30 to 60 minutes after the injection of morphine. The animals became quiet and easy to handle; the dilatation of the pupils subsided within less than 15 minutes.

Acute toxicity was determined in mice (total 400 animals) following subcutaneous injection. N-allyl-normorphine did not produce the characteristic manifestations of morphine poisoning in mice, such as restlessness and "S" shaped tails. The respiration gradually became labored and irregular and all animals receiving lethal doses died of respiratory failure. The heart was found beating after the respiration had ceased. Lethal doses caused deaths within 15 to 90 minutes, whereas deaths following corresponding doses of morphine occurred within 45 minutes to 3 hours after the subcutaneous injection. In

TABLE 1

Acute toxicity (L.D. 50) in mgm. per kgm. following subcutaneous injection

N-ALLYL NORMORPHINE	MORPHINE SULFATE	MORPHINE SULFATE FOLLOWING PREMEDICATION WITH N-ALLYL-NORMORPHINE
670	660	820

TABLE 2

Effect of N-allyl-normorphine on the mortality of mice injected subcutaneously with 800 mgm. per kgm. of morphine

N-ALLYL-NORMORPHINE DOSE	NUMBER OF ANIMALS	MORTALITY
None (control)......	50	88%
2 × 150 mgm. per kgm. 15 min. and 45 min. after morphine	25	40%
3 × 100 mgm. per kgm. 15 min., 45 min. and 105 min. after morphine...............	25	28%

mice, as demonstrated by the comparison of the L.D. 50 of both substances in table 1, the toxicity of N-allyl-normorphine was about the same as that of morphine.

Administration of N-allyl-normorphine made the animals more resistant to the toxic effects of morphine. This was demonstrated by the determination of the toxicity of morphine in mice which received 250 mgm. per kgm. of N-allyl-normorphine prior to the injection of morphine. As shown in table 1, in animals treated with N-allyl-normorphine, the L.D. 50 of morphine rose from 660 mgm. to 820 mgm. per kgm.

The effectiveness of N-allyl-normorphine in counteracting morphine poisoning was studied in mice which had received lethal doses of morphine (table 2). A dose of 800 mgm. per kgm. of morphine was fatal to 88% of the mice, deaths occurring from 45 minutes to two and one-half hours after the injection. In these animals restlessness and irritability were promptly suppressed by N-allyl-

normorphine and the animals remained quiet for a period of about one hour after which the symptoms of morphine poisoning again became evident. When restlessness reappeared, a subsequent injection of N-allyl-normorphine again produced its antagonistic effect. Since the effect of N-allyl-normorphine is of shorter duration than that of morphine it seemed advisable to administer N-allyl-normorphine in divided doses at regular intervals in these experiments. Whereas a single dose of 400 mgm. per kgm. of N-allyl-normorphine did not significantly increase the incidence of survival in these mice, the repeated administration of N-allyl-normorphine in doses of 100 and 150 mgm. per kgm. at intervals as indicated in table 2 caused a reduction in the mortality of morphine from 88% to 40% and 28% respectively.
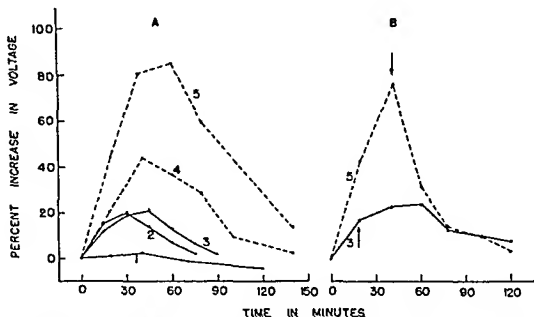
Fig. 1. Effect of N-Allyl-Normorphine and of Morphine on the Threshold for Pain Perception in Mice. Each Curve Represents the Average Response of 15 Mice

Analgesic Effect. The analgesic effect of N-allyl-normorphine was studied in mice by determining the threshold for pain perception in the abdominal skin following electrical shocks of known voltage. The substance was injected subcutaneously in doses ranging from 10 to 200 mgm. per kgm. to groups of 15 mice. No effect was noted following the injection of 10 mgm. per kgm. Doses of 50 and 100 mgm. per kgm. raised the threshold for pain perception by about 20%, and this effect lasted for about one hour. No further decrease in pain perception was obtained by increasing the dose of N-allyl-normorphine to 200 mgm. per kgm. Under the same experimental conditions, morphine sulfate (5 and 10 mgm. per kgm. respectively) raised the pain threshold by about 40 and 80% respectively. The analgesic effect of morphine lasted from 2 to 3 hours. The results summarized in Figure 1 clearly demonstrate that N-allyl-normorphine is much less effective than morphine in raising the threshold for pain.

When morphine was given to mice which had received N-allyl-normophine 20 minutes before, no further increase in the analgesic effect was obtained (figure 1, Curve B at ↑). When morphine was given first and the N-allyl-normorphine injected 40 minutes afterwards the analgesic effect of morphine was rapidly abolished and the threshold for pain perception reduced to the level obtained by N-allyl-normorphine alone (figure 1, Curve B at ↓).

Circulation. In rabbits anesthetized with urethane (1 gram per kgm. subcutaneously) intravenous injection of N-allyl-normorphine was found to produce a transient depression of the blood pressure. A slight decrease was noticeable after the injection of 2 mgm. per kgm.; it became more pronounced (up to 40 mm. Hg) following 5, 10 or 20 mgm. per kgm., respectively. The effect lasted for about 1 to 3 minutes, depending on the dose of N-allyl-normorphine. Corresponding doses of morphine were less effective in lowering the blood pressure.
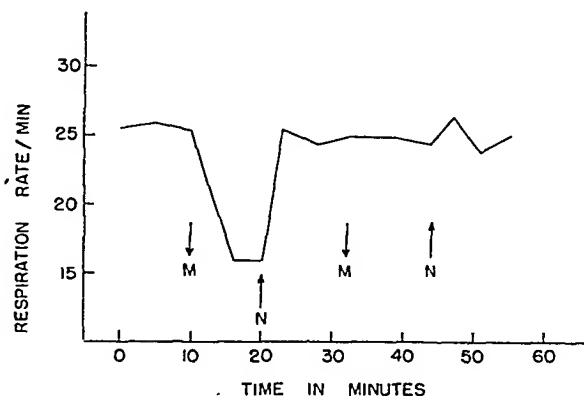
FIG. 2. EFFECT OF N-ALLYL-NORMORPHINE AND OF MORPHINE UPON THE RESPIRATION
Rabbit 2.9 kgm. anesthetized with urethane (1 gram per kgm.).
At M intravenous injection of morphine (2 mgm. per kgm.). At N intravenous injection of N-allyl-normorphine (2 mgm. per kgm.).

Respiration. In 12 experiments the respiratory rate of rabbits anesthetized with urethane and of non-anesthetized rabbits was not significantly influenced by intravenous injections of 2 to 20 mgm. per kgm. of N-allyl-normorphine, except for a slight transient acceleration concomitant with the fall in the blood pressure. Injections of 50 mgm. per kgm. caused death by respiratory failure.

After the administration of N-allyl-normorphine, morphine failed to depress the respiration even when given intravenously in very large doses (20 mgm. per kgm.) in other experiments in which morphine, given before the animal received N-allyl-normorphine, produced a marked depression of the respiration, the subsequent injection of N-allyl-normorphine immediately restored the respiratory rate to the previous level. The prompt counter-effect of N-allyl-normorphine is illustrated in Figure 2 which also shows the inffectiveness of morphine subsequent to the administration of N-allyl-normorphine.

Influence upon the Effect of Apomorphine. The question whether *N*-allyl-normorphine prevents the effects of apomorphine was studied in rabbits and dogs. Rabbits receiving 1 mgm. per kgm. of apomorphine hydrochloride twenty minutes after the injection of 10 mgm. per kgm. of *N*-allyl-normorphine became very excited and restless; the manifestations of apomorphine intoxication in these animals were not influenced by the administration of *N*-allyl-normorphine. Likewise no antagonistic effect was observed in dogs, for vomiting followed the injection of apomorphine regardless whether the animals had previously received 10 mgm. per kgm. of *N*-allyl-normorphine or not.

SUMMARY

A comparison of the effects obtained with *N*-allyl-normorphine and with morphine shows that *N*-allyl-normorphine is about as toxic, but much less effective than morphine in raising the threshold for pain in mice. In contrast to morphine small doses do not depress the respiration; very large doses, however, cause arrest of the respiration, and all animals succumbing to *N*-allyl-normorphine die of respiratory failure.

N-allyl-normorphine prevents or abolishes the action of morphine. Premedication with this substance increases the resistance of mice to morphine so that otherwise lethal doses are tolerated without untoward effects. Furthermore, an injection of *N*-allyl-normorphine prior to that of morphine prevents analgesia (mice), respiratory depression (rabbits), and the general depressing (dogs) and stimulatory (cats) effects as well.

If morphine is administered in the first place, a subsequent injection of *N*-allyl-normorphine abolishes its analgesic effect (mice) restores the depressed respiration to its previous level (rabbits) and abolishes its toxic manifestations in cats and dogs. The reduction in the mortality of morphine poisoning in mice by *N*-allyl-normorphine further demonstrates the effectiveness of this substance as an antidote against morphine.

Since the site of action of morphine is generally assumed to be central, it would appear that *N*-allyl-normorphine by virtue of its chemical relationship to morphine exerts its action upon the same centers as morphine rendering them less sensitive to morphine. This assumption would offer the most plausible explanation for the observations reported.

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A COMPARISON OF THE MECHANISM OF ACTION OF ARSENICALS AND SULFONAMIDES

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A decrease in the oxygen consumption of *E. coli* in a synthetic medium was found by Hirsch (1) to be caused by high concentrations (1.10^{-1}M) of sodium *p*-aminophenylarsonate (atoxyl), an effect which was antagonized by *p*-aminobenzoic acid (*PAB*). Such an antagonism was suggested on theoretical grounds by Bell and Roblin (2), on the basis of the structural similarity between atoxyl and PAB. The chemotherapeutic activity of certain trivalent arsenicals against staphylococci and some strains of *Strep. viridans* has been investigated by Osgood (3, 4). In this investigation attention has been given to the possibility of a relationship between the mechanism of action of sulfonamides and arsenicals on micro-organisms.

EXPERIMENTAL STUDIES. Complete inhibition of visible growth of *E. coli* by atoxyl was obtained in the simple salt-glucose medium described by Tsuchiya *et al.* (5). Tubes containing the test substances dissolved in the medium (5.0 cc.) were inoculated with a 1:1000 dilution (0.1 cc.) of a 24 hour culture of *E. coli* in the same medium; the inoculum contained 50,000 to 100,000 organisms, as determined by dilution counts. The strain of *E. coli* was of fecal origin and had no previous contact with sulfonamides. The pH of the medium, after autoclaving, was 7.0 to 7.2 and was unaltered by the materials tested. Turbidity measurements were made in a Summerson-Klett photoelectric colorimeter (540 filter) when the control tubes had just attained maximum turbidity (a reading of 90 to 120 on the log scale of the instrument). Color controls, containing no organisms, were read simultaneously. The time at which maximum turbidity was reached varied somewhat from time to time, and accordingly readings were not made at a constant time interval.

Table 1 shows that atoxyl or sulfanilamide will produce complete inhibition of visible growth of *E. coli* and that this effect is antagonized completely by *PAB*. The *PAB:atoxyl* ratio was much smaller than that of *PAB:sulf*. *PAB* accelerated slightly the rate of growth of the organism but did not increase the maximum turbidity attained (table 2). A yellow to light brown color, which rendered accurate turbidimetric reading impossible, was consistently produced in those tubes in which a heavy growth of bacteria had taken place in the presence of a concentration of atoxyl greater than the minimum required to inhibit growth and a concentration of *PAB* which completely neutralized the effect of the atoxyl. Turbidities in such tubes could be compared only by visual observation, as indicated in table 1. Methyl *m*-amino-*p*-hydroxybenzoate ('Orthoform')[1] ac-

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[1] Winthrop Chemical Co. has applied its trademark 'Orthoform' to methyl *m*-amino-*p*-hydroxybenzoate.

TABLE 1

Antagonism of the bacteriostatic activity of atoxyl and of sulfanilamide on E coli by p-aminobenzoic acid in a salt-glucose medium

DRUG	PAB	READING*	RATIO PAB DRUG
0	0	119(4+)†	
Atoxyl 2×10^{-3}M	0	2	
Atoxyl 2×10^{-3}M	1×10^{-6}M	(4+)	1 2,000
Atoxyl 2×10^{-3}M	1×10^{-7}M	9	1 20,000
Atoxyl 1×10^{-3}M	0	3	
Atoxyl 1×10^{-3}M	1×10^{-6}M	(4+)	1 1,000
Atoxyl 1×10^{-3}M	1×10^{-7}M	55	1 10,000
Atoxyl 5×10^{-3}M	0	2 5	
Atoxyl 5×10^{-3}M	1×10^{-6}M	(4+)	1 5,000
Atoxyl 5×10^{-3}M	1×10^{-7}M	2	1 50,000
Atoxyl 1×10^{-3}M	0	0	
Atoxyl 1×10^{-3}M	1×10^{-7}M	124	1 10,000
Atoxyl 1×10^{-3}M	1×10^{-8}M	53	1 100,000
Atoxyl 5×10^{-6}M	0	110	
Sulfanilamide 5×10^{-6}M	0	3	
Sulfanilamide 5×10^{-6}M	1×10^{-6}M	121	1 50
Sulfanilamide 5×10^{-6}M	1×10^{-7}M	94	1 500
Sulfanilamide 2 5×10^{-6}M	0	28	
0	0	109	
Atoxyl 1×10^{-3}M	0	9	
Atoxyl 1×10^{-3}M	5×10^{-7}	122	1 2,000
Atoxyl 1×10^{-3}M	1×10^{-7}	84	1 10,000
Atoxyl 1×10^{-3}M	5×10^{-8}	43	1 20,000
Atoxyl 1×10^{-3}M	1×10^{-8}	9	1 100,000
Atoxyl 1×10^{-3}M	5×10^{-9}	3	1 200,000
Sulfanilamide 5×10^{-6}M	0	0	
Sulfanilamide 5×10^{-6}M	5×10^{-7}	114	1 100
Sulfanilamide 5×10^{-6}M	1×10^{-7}	50	1 500
Sulfanilamide 5×10^{-6}M	5×10^{-8}	16	1 1,000
Sulfanilamide 5×10^{-6}M	1×10^{-8}	16	1 5,000
Sulfanilamide 5×10^{-6}M	5×10^{-9}	3	1 10,000

* Direct readings from log scale of Summerson-Klett photoelectric colorimeter

† (4+) designates maximal turbidity as judged by visual observations

celerated the growth of *E coli* to approximately the same extent as did *PAB*, but the bacteriostasis caused by atoxyl and by sulfanilamide was not antagonised o-Aminobenzoic acid and benzoic acid likewise were not antagonists

to atoxyl and sulfanilamide bacteriostasis. Procaine caused a complete reversal
of bacteriostasis. Methionine also reversed completely the bacteriostatic action
of atoxyl and sulfanilamide (table 3). However, the marked accelerating effect
of methionine on the rate of growth of this strain of *E. coli* in the medium used
(table 2) rendered difficult an interpretation of the exact degree of true
antagonism.

The relative bacteriostatic activity of various arsenicals and sulfonamides is
shown in table 4. The well-established importance of the free *p*-amino group to
sulfonamide action seems to find its analogy in the arsonic acids, since only the
p-amino compound amongst those tested, is bacteriostatic. The findings

TABLE 2

Acceleration of the growth rate of E. coli by PAB and by methionine in salt-glucose medium

	TIME (HOURS)					
	16½	18	19	20	21	24
Control	31	60	72	94	108	125
PAB 1×10⁻⁷M	35	85	107	118	130	128
Methionine 1×10⁻⁷M	87	111	124	121	130	132

TABLE 3

Antagonism of the bacteriostatic action of atoxyl and sulfanilamide by methionine and by PAB

	NO ANTAGONIST	PAB 1 × 10⁻⁷M	METH 1 × 10⁻⁷M
No drug	97	108	108
Atoxyl 2×10⁻³M	0	26	21
1×10⁻³M	15	115	119
Sulfanilamide 1×10⁻⁴M	2	17	31
5×10⁻⁵M	2	84	102

strongly indicate a common mechanism for the bacteriostatic action of sulfon-
amides and *p*-aminophenylarsonic acid.

The trypanocidal activity of atoxyl, although not demonstrable in a medium
consisting of 1 part serum and 2 parts Locke's solution, is easily seen in a similar
medium containing whole rat blood instead of serum (table 5). That this ac-
tivity is due to, and takes place only after, reduction to the arsenoxide (—As=O)
form has been established (6). Table 5 shows that the trypanocidal activity, as
measured by the method of Wright and Peters (7), is not altered by PAB.
Similar results were recently reported by Sandground (8) in *in vivo* experiments.
Acetarsone was found to be trypanocidal in the same concentrations as atoxyl,
with or without PAB. Tryparsamide has also been found to be trypanocidal in
such a medium (6). Thus the trypanocidal activity of the phenylarsonic acids
appears to be exerted through a different mechanism than their bacteriostatic
action.

Although 'Mapharsen'[3] was more bacteriostatic for *E coli* than atoxyl (table 4) its arsonate analogue was inactive Its action was not antagonized by PAB, by 'Orthoform ' or by methionine 'Mapharsen' was also found to be highly bacteriostatic for *L casei* (0 3 mgm per 100 cc) in the very adequate medium described by Landy and Dicken (9), this effect being unaltered by PAB and by Ortho

TABLE 4

Minimum concentrations of various sulfonamides and arsenicals preventing visible growth of E coli in salt glucose medium

DRUG	MINIMUM BACTERIOSTATIC CONC	
	Molarity	Approximate dilution
Sodium *p* aminophenylarsonate (Atoxyl)	1×10^{-2}M	1 4 600
Sodium *N* phenyl glycinamide *p* arsonate (Tryparsamide)	Non bacteriostatic	1 100
Sodium *m* acetylamino *p* hydroxyphenylarsonate (Acetarsone)	Non bacteriostatic	1 100
Sodium *m* amino *p* hydroxy phenylarsonate[2]	Non bacteriostatic	1 200
m Amino *p* hydroxyphenylarsenoxide (Mapharsen)	1×10^{-5}M	1 38 700
Sulfanilamide	5×10^{-3}M	1 120 000
Sulfadiazine	5×10^{-7}M	1 8 000 000
m Amino *p* hydroxybenzenesulfonamide	1×10^{-3}M	1 550
m Nitro *p* hydroxybenzenesulfonamide	2 5$\times 10^{-5}$M	1 18 400

TABLE 5

Minimum concentration of atoxyl and of 'mapharsen which when incubated with Trypanosoma equiperdum for 18 hours at 37°C in a blood Locke s solution medium renders the organisms non infective for young rats

ATOXYL		MAPHARSEN		
Alone	In the presence of PAB (2 mgm per 100 cc)	Alone	In the presence of orthoform (2 mgm per 100 cc)	In the presence of PAB (2 mgm per 100 cc)
1 0 05 m*	1 0 05 m	1 5 m	1 10 m	
1 0 05 m	1 0 05 m	1 10 m	1 5 m	1 5 m
1 0 05 m	1 0 05 m	1 5 m	1 5 m	

* m = millions

form ' Snell and Mitchell (10) found this organism to be very resistant to the effect of sulfonamides in a similar medium the bacteriostatic action of high concentrations being readily reversed by *PAB* These findings indicate that 'Mapharsen and the sulfonamides have quite different points of attack

[2] The *m* amino *p* phdroxy benzoic acid used in this test was generously supplied by Dr G W Raiziss

[3] Parke Davis and Co has applied its trademark 'Mapharsen' to *m* amino *p* hydroxy phenylarsenoxide

This difference is further demonstrated by comparing the action of 'Mapharsen' and sulfonamides on trypanosomes. 'Mapharsen' is highly trypanocidal (table 5), and its trypanocidal activity is not significantly altered by *PAB* or 'Orthoform.' Sulfanilamide is not trypanocidal *in vivo* (11) and we have been unable to influence the course of *Trypanosoma equiperdum* infection in rats and mice with a highly active, slowly excreted sulfonamide, sulfamerazine (*2*-sulfanilamido-*4*-methylpyrimidine) (12). This was true even when concentrations of 30 mgm. of sulfamerazine per 100 cc. of blood were present at the time of infection and were maintained until the death of the animals. Furthermore, the well-established, favorable effect of the introduction of the *m*-amino-*p*-hydroxy groupings on the trypanocidal and spirocheticidal action of phenylarsenoxide does not apply to sulfonamides since we have found *m*-amino-*p*-hydroxybenzenesulfonamide, as well as its nitro analogue, to be non-trypanocidal *in vitro*; this is also the case *in vivo*, when the drugs are fed at a level of 0.5 per cent in the diet. *m*-Amino-*p*-hydroxybenzenesulfonamide is practically non-bacteriostatic (table 4), while the nitro analogue is somewhat more active, but both are unaffected by PAB or by 'Orthoform.'

SUMMARY

The mode of action of sodium *p*-aminophenylarsonate (atoxyl) on *E. coli* appears to be similar to that of sulfonamides, since the same mechanisms of antagonism appear to be involved.

The bacteriostatic and trypanocidal activities of *m*-amino-*p*-hydroxyphenylarsenoxide, 'Mapharsen,' apparently are exerted through a different mechanism than that which is involved in the bacteriostatic action of sulfonamides.

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THE EFFECT OF OXYGEN, SOYBEAN LECITHIN, CARBAMYL CHOLINE, AND FURFURYL TRIMETHYL AMMONIUM IODIDE ON EXPERIMENTAL POLYCYTHEMIA[1]

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In previously reported experiments (1–4) we have shown that raw liver, choline, certain choline esters and ethers and certain vasodilator drugs are capable of depressing experimental polycythemia in dogs and rabbits The methods used for inducing polycythemia included daily exposure of animals to low atmospheric pressure, daily physical exercise, administration of cobaltous chloride, ephedrine or benzedrine The depression of polycythemia is apparently unaccompanied by any dilution of the blood, and it occurs in splenectomized as well as in normal animals The depressant drugs probably act by producing vasodilation and thereby increasing the blood and oxygen supply to red bone marrow We have performed the reverse experiment of inducing polycythemia by the continued daily administration of vasoconstrictor drugs (ephedrine, epinephrine and posterior pituitary) to dogs and rabbits (5–6)

Theoretical considerations would indicate that if our concept of the mechanism of polycythemia depression is correct, we should be able to depress polycythemia by increasing the oxygen content of the arterial blood The investigation herein reported shows the results of the administration (by inhalation) of an atmosphere of pure oxygen to polycythemic dogs for one hour daily We also report the results of the daily administration of soybean lecithin, carbaminoyl choline and furfuryl trimethyl ammonium iodide to polycythemic dogs

PROCEDURE Experimental polycythemia was produced in three dogs by the daily subcutaneous administration of five units of solution of posterior pituitary or pitressin and in two dogs by the daily oral administration of three mgm per kgm of cobalt as the chloride salt Two of the dogs had been splenectomized 6 months prior to the commencement of these experiments All animals were maintained on a constant adequate diet and were allowed water *ad libitum*

After the establishment of polycythemia which required two or three weeks 3 dogs receiving pituitrin and 2 dogs receiving cobalt were given commercial soybean lecithin[2] in a daily oral dose of 3 grams

Subsequently carbaminoyl choline chloride[3] was administered subcutaneously to 4 polycythemic dogs in a total daily dose of 0 1 mgm Furmethide (furfuryl trimethyl

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ammonium iodide)[4] was administered to 3 dogs with pituitrin-induced polycythemia in a daily subcutaneous dose of 5 mgm (about 0 5 mgm per kgm).

Pure oxygen was administered for one hour daily to 3 dogs having pituitrin-induced polycythemia   The animals breathed the oxygen in a closed system consisting of a tight nosepiece connected with a Sanborn basal metabolism apparatus   The expired carbon dioxide was absorbed by soda lime, while the consumed oxygen was replaced by a steady flow of the pure gas into the metabolism machine

FIG. 1 (left) DEPRESSION OF PITUITRIN-INDUCED POLYCYTHEMIA IN 3 DOGS, BY THE INHALATION OF 100% OXYGEN FOR ONE HOUR DAILY

Dashed lines indicate oxygen administration   Figures at start of each line indicate the degree of polycythemia for each animal in terms of percentage increase of erythrocyte count above normal Spl = splenectomized dog

FIG. 2 (right). EFFECT OF DAILY ORAL ADMINISTRATION OF 3 GRAMS OF SOYBEAN LECITHIN ON EXPERIMENTAL POLYCYTHEMIA

$P P$ = dog receiving posterior pituitary injections, C = cobalt fed dog

Blood samples were drawn from the external saphenous veins of the dogs while they were blindfolded and in a quite, unexcited state, at least 18 hours after any previous experimental procedure or medication   Red cell counts and hemoglobin percentages (Hellige) were determined at frequent intervals throughout the experiments, while total leukocyte counts were made occasionally

RESULTS,   The administration by inhalation of pure oxygen to 3 polycythemic dogs for one hour daily caused prompt reductions of their erythrocyte counts (figure 1) and hemoglobin percentages, in spite of continued daily injections of pituitrin or pitressin   The depression of the polycythemia persisted for the 5 or 6 days during which 100% oxygen was administered, and cessation of oxygen administration was followed in 3 or 4 days by a return of the polycythemia

[4] Furmethide was supplied by the Smith, Kline, and French Laboratories, Philadelphia, Pa.

Total leukocyte counts remained fairly constant throughout the experiment One of the animals had been splenectomized six months prior to this investigation

Figure 2 shows the results of the daily oral administration of 3 grams of soy bean lecithin to 2 dogs having cobalt polycythemia and 3 dogs with pituitrin induced polycythemia It will be noted that this procedure caused gradual reductions in the red cell counts of all 5 dogs, which persisted throughout the period of lecithin feeding in spite of continued hemopoietic stimulation by cobalt or pituitrin Hemoglobin percentages (not shown) varied proportionately with the erythrocyte counts, but leukocyte counts showed no uniform change and remained fairly constant Upon cessation of lecithin administration, poly
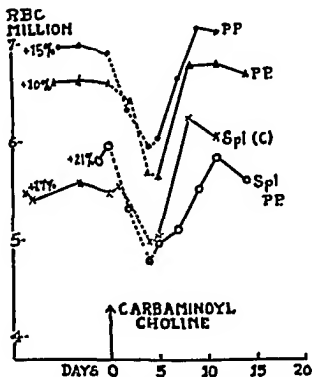
FIG 3 DEPRESSION OF POLYCYTHEMIA BY CARBAMINOYL CHOLINE CHLORIDE

Dashed lines indicate time during which drug was administered Figures at left of each line show degree of polycythemia for each animal as per cent increase of erythrocytes over normal

$P\ P$ = posterior pituitary injected dogs C = cobalt fed dog Spl = splenectomized dog

cythemia returned to all dogs within about 4 days Two of the dogs used in this experiment had been splenectomized previously

The subcutaneous administration of a total daily dose of 0 1 mgm of carbaminoyl choline chloride to 3 dogs with pituitrin induced polycythemia and one cobalt fed dog produced significant depressions in their red cell counts (figure 3) and hemoglobin percentages within 4 days Upon cessation of Doryl injections the erythrocyte numbers of these animals gradually returned to their polycythemic levels Leukocyte counts remained fairly constant throughout these procedures Two of the dogs had been previously splenectomized

One splenectomized and two normal dogs having pituitrin induced polycythemia were given daily subcutaneous injections of furmethide (furfuryl tri

ammonium iodide)[4] was administered to 3 dogs with pituitrin-induced polycythemia in a daily subcutaneous dose of 5 mgm. (about 0.5 mgm. per kgm.).

Pure oxygen was administered for one hour daily to 3 dogs having pituitrin-induced polycythemia. The animals breathed the oxygen in a closed system consisting of a tight nosepiece connected with a Sanborn basal metabolism apparatus. The expired carbon dioxide was absorbed by soda lime, while the consumed oxygen was replaced by a steady flow of the pure gas into the metabolism machine.
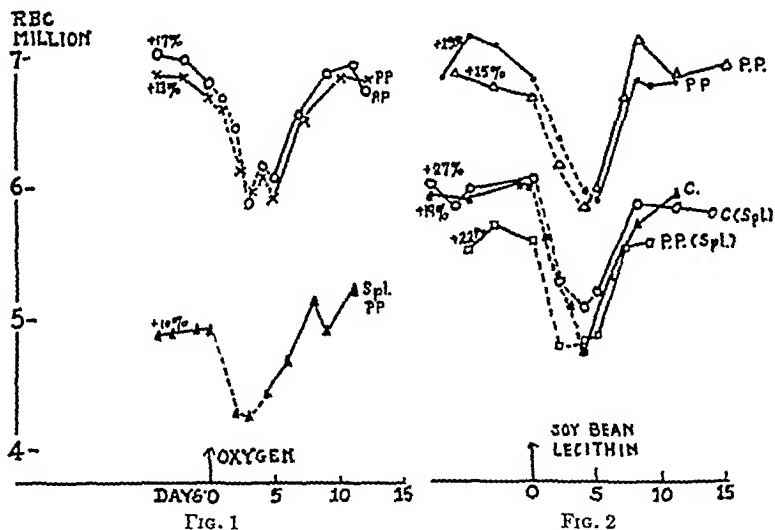

FIG. 1 (left). DEPRESSION OF PITUITRIN-INDUCED POLYCYTHEMIA IN 3 DOGS, BY THE INHALATION OF 100% OXYGEN FOR ONE HOUR DAILY

Dashed lines indicate oxygen administration. Figures at start of each line indicate the degree of polycythemia for each animal in terms of percentage increase of erythrocyte count above normal. *Spl.* = splenectomized dog.

FIG. 2 (right). EFFECT OF DAILY ORAL ADMINISTRATION OF 3 GRAMS OF SOYBEAN LECITHIN ON EXPERIMENTAL POLYCYTHEMIA

P.P. = dog receiving posterior pituitary injections; *C* = cobalt fed dog.

Blood samples were drawn from the external saphenous veins of the dogs while they were blindfolded and in a quite, unexcited state, at least 18 hours after any previous experimental procedure or medication. Red cell counts and hemoglobin percentages (Hellige) were determined at frequent intervals throughout the experiments, while total leukocyte counts were made occasionally.

RESULTS. The administration by inhalation of pure oxygen to 3 polycythemic dogs for one hour daily caused prompt reductions of their erythrocyte counts (figure 1) and hemoglobin percentages, in spite of continued daily injections of pituitrin or pitressin. The depression of the polycythemia persisted for the 5 or 6 days during which 100% oxygen was administered, and cessation of oxygen administration was followed in 3 or 4 days by a return of the polycythemia.

‘ Furmethide was supplied by the Smith, Kline, and French Laboratories, Philadelphia, Pa.

Commercial soybean lecithin, which reduced polycythemia in our experiments (figure 2), probably did so by virtue of its choline content which is reported to be about 3% (8) presumably in an available form. We have previously reported that purified egg lecithin in a daily oral dose of 1 gram is without effect on experimental polycythemia (3).

It is not surprising that carbamyl choline chloride depressed polycythemia in our dogs, since we have shown previously (3, 4) that choline and acetyl beta-methyl choline, which also produce vasodilation, are effective against polycythemia.

Furfuryl trimethyl ammonium iodide was found by Fellows and Livingston (9) to exert parasympathomimetic actions, some of which are blocked by atropine. Since it was shown to produce vasodilation, we tried it on our polycythemic dogs and found that it was capable of reducing their red cell counts (figure 4).

It should be emphasized that total leukocyte counts showed no constant change either during the production or depression of polycythemia, and that splenectomized dogs responded in exactly the same manner as normal dogs.

CONCLUSIONS

Exposure for one hour daily to an atmosphere of 100% oxygen caused significant reductions in the basal erythrocyte numbers of 3 dogs with pituitrin-induced polycythemia in spite of continued daily injections of pituitrin. One of the animals had been splenectomized long before this experiment.

The daily oral administration of 3 grams of soybean lecithin to 5 polycythemic dogs caused significant reductions in their erythrocyte counts, with no appreciable change in leukocyte counts, in spite of continuation of hemopoietic stimulating measures.

Carbamyl choline chloride, in a daily subcutaneous dose of 0.1 mgm., depressed experimental polycythemia in 2 splenectomized and 2 normal dogs in spite of continued daily procedures used to produce the polycythemia (i.e., cobalt or pituitrin administration).

Furfuryl trimethyl ammonium iodide likewise depressed "pituitrin polycythemia."

These experimental results indicate that the agents used to depress polycythemia probably act by increasing the oxygen supply to the red bone marrow, thereby diminishing the local hypoxia which is probably the stimulus to the development of polycythemia.

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INFLUENCE OF VARIOUS DRUGS ON THE THRESHOLD FOR ELECTRICAL CONVULSIONS[1]

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Methods of producing convulsions by passing electrical currents through the brain in unoperated animals have been described by Spiegel (1) and Putnam and Merritt (2) among others, and have become important procedures in treating certain psychiatric conditions as a "shock" therapy. Spiegel observed that chloral and phenobarbital raised the threshold, and Merritt and Putnam (3) reported on a considerable series of compounds which, however, were for the most part tested at only a single dosage level. It appeared important to investigate some of the factors controlling the reactivity of the brain to this type of stimulus, and particularly whether the excitability could be modified by drugs. It is often assumed that the convulsive threshold is governed by the excitability of the cerebral cortex, although this does not seem to be clearly proven. Originally we intended to use this method for further analysis of the centrally acting amines, but eventually included representative groups of drugs, determining the relationship of dosage to effect over considerable ranges of tolerated doses. This report presents the results obtained.

METHODS. Unanesthetised rabbits were used throughout, the same animals being used repeatedly at intervals of from 4 days to 2 weeks. However, if any animal became erratic in response, lost weight, or showed other evidence of ill health, it was replaced by a fresh one. In general 7 rabbits were used in a test group, a smaller number being used only for special purposes, or in case of depletion by death or intercurrent infection.

After many tests with various methods, the following standard procedure was adopted. There was put in the mouth of the rabbit a metal bit made from an iron nail, soldered near the ends of which were circular metal discs so it could not slip out the side of the mouth. The hair between the ears was clipped short and a rubber sponge electrode wet with salt solution was applied to the skin between the ears and held in place by a metal plate placed over it. This plate was 20 mm. in diameter and had two tabs turned upwards at the edges. The two electrodes were held firmly in place by rubber bands on each side snapped over the tabs and the bit, and including the ears. This kept the electrode from being moved out of position, by struggling or deliberate attempts at removal by the animal.

A special stimulator was built with high fixed resistance on the output side to make negligible any fluctuations in current flow because of variations in skin resistance. Resistance of the tissues of the rabbit, under the conditions, fluctuated between 100 and 500 ohms. By putting 10,000 ohms in the output circuit the relatively small variations from the tissues had negligible effects on the current flow.

As a stimulating current the 110 volt 60 cycle current of the laboratory was used   This has a sine wave form   As is depicted diagrammatically in figure 1, this current was fed through a snap switch into a continuously variable auto transformer, for varying the voltage   The output of this was fed into a fixed radio transformer giving a voltage step up of 1 to 10   From this the current passed through one of a pair of a c milliammeters with 6 inch scales from which the current flow was read   The current then passed through the fixed resistors to flexible wire leads which attached to the two electrodes by alligator clamps

The test procedure was to put the electrodes on each of a group of rabbits and to determine the amount of current flow required to produce epileptiform convulsions   The transformer was set to give the voltage required for the desired milliamperes of current and the stimulus applied for exactly 15 seconds   At currents below the true threshold the flow through the motor cortex caused muscular movements which were readily distinguished from the epileptiform convulsions   The former were tonic synchronous with the current and did not spread   They started and stopped exactly with the current flow   The true epileptiform convulsions sometimes began while the current was still on but often did not develop for as long as 2 minutes after it had stopped   These were clonic, radiating in distribution, were accompanied by postural eccentricities and left the animal depressed,
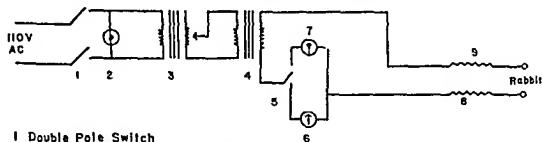
I Double Pole Switch
2 IIOV Pilot Light Dialco Model 910 CD
3 Variable Transformer Varitran Model I 0-130 Volts 5 0 Amp Max
4 Fixed Transformer T19P54 Plate Supply Transformer Thordorson (Connected For II20 Volts Output)
5 Single Pole Double Throw Switch
6 Milliammeter 10-100 Milliamperes Triplett Model 735
7 Milliammeter 0-25 Milliamperes Triplett Model 735
8-9 Two 5000 Ohm Resistances IRC Resistors Each 5000 Ohms Type HA

FIG 1 DIAGRAM OF THE HIGH RESISTANCE STIMULATOR USED TO DETERMINE THE THRESHOLD OF THE CEREBRAL CORTEX FOR EPILEPTIFORM CONVULSIONS

ataxic, or even apparently mentally confused so that they would fall off a table or bump into objects in hopping   These convulsions did not stop when the current was switched off   There was always cyanosis from interference with respiration and asphyxia undoubtedly contributed to the terminal seizures

The 15 second duration of stimulation was long enough to be practically infinite in comparison to the chronaxie   If a short time interval had been used, such as the 0 1 to 0 5 second in human shock therapy, the duration of the stimulus might have affected threshold intensity, particularly when the reactivity of the brain had been modified by drug action   Longer stimuli than this were found to be somewhat depressant and, through fatigue, tended to produce abnormally high thresholds   With shorter time intervals the results were more irregular, and there was greater difficulty in controlling accurately the current flow and duration

The procedure of a determination was first to test each rabbit of a group with a current about 4 ma below the expected convulsive threshold   After a 5 minute interval, the entire group was tested again with a current 2 ma higher   This was continued with successively greater current flows until the current required to cause the characteristic convulsions of each rabbit had been determined   When the required end point for an animal was reached, it was no longer stimulated that day   At least 5 minutes of rest intervened between suc

cessive stimulations in any animal, since stimulation at shorter intervals raised the threshold through fatigue.

Each animal of a group was measured first without medication about 10 times during several weeks, until the threshold values were regular without progressive shift in value. The individual figures only infrequently deviated widely from the average value for that rabbit, the extreme control thresholds observed in any determination being 16 and 30 ma. The average control threshold for the entire group was 21.0 ma. which varied for individual rabbits between an average of 17.9 and 25.6 ma. These mean thresholds had an average standard error of 0.73 ma. or about 3.5 per cent of the threshold value. The variability of the values was not reduced by expressing the changes as percentages of the controls, so the simpler absolute values were used for the calculations throughout. After the control thresholds had been satisfactorily established the drug was administered and the threshold values re-determined at a time to coincide with its peak of action. Several days later the same group of animals were tested again without medication as another control and check on the disappearance of the drug-action. The mean threshold value for all the control runs on a given animal was subtracted from that obtained under the influence of the drug to give the change in threshold observed. These changes from the individual animals were averaged and the standard error of the mean calculated. According to the "t" distribution, the change should be 2.8 times its standard error for 95 per cent reliability for 5 animals, 2.6 times for 6 animals, and 2.4 times for 7 (4). The doses were doubled successively in subsequent tests until either the maximal tolerated dose was approached as judged by symptoms, or some deaths were caused. If the first dose caused a definite change, lower doses were also tested by successively halving the previous dose until ineffective levels were reached. All the doses were given per kilogram of body weight, and are so expressed in this paper.

The mass of data accumulated has been condensed in tables 1 to 5 for sake of brevity to give only the salient points. The drugs used have been grouped according to predominant physiological actions, and the results are discussed briefly.

Barbitals and related compounds. In table 1 are summarized the data on three barbitals, two hydantoins, and propazone. It can be seen that these compounds all raised the convulsive thresholds to a very considerable degree. If corresponding doses of the barbitals are compared, it will be seen that there was remarkably little difference between them. Dilantin failed to show the great decrease in excitability ascribed to it by Merritt and Putnam (3) at a low dosage, although it had clear-cut effects in higher doses. Since these investigators used cats, this minor discrepancy may arise from delayed or incomplete gastric absorption in rabbits used by us, or there may be other species differences. Another hydantoin derivative[2] was similar in potency to dilantin and required approximately the same range of dosage. Chemically, propazone is an oxazolidinedione with narcotic and hypnotic properties (5). This compound also raised the convulsive threshold, but not to a degree comparable to the barbitals, even after the highest doses.

The results with this group of products are quite consistent in demonstrating a decrease in the excitability of the brain which, for most of them, depends on the size of the dose administered. The choice between them for antagonizing epileptic attacks would depend, in part at least, on the nature and extent of

[1] Prepared by the Sandoz Chemical Works, Inc.

their side effects, such as hypnosis, tissue changes, etc , especially after continued administration

Other central depressants The results with a miscellaneous group of other central depressants are summarized in table 2

TABLE 1

Effects of barbitals and related compounds on convulsive thresholds

DRUG	DOSE PER KG	NO OF RABBITS USED	MEAN CHANGE IN THRESHOLD	STANDARD ERROR OF MEAN	NO OF RABBITS DIED	METHOD OF ADMINISTRATION
	mgm		*ma*	*ma*		
Pentobarbital sodium	2 5	14	+1 98	±0 81		Subcutaneously 30 min before
	5 0	14	+2 76	±0 74		
	10	7	+3 7	±1 01		
	20	7	+0 98	±1 08		
Phenobarbital sodium	1 5	6	+1 55	±0 81		Subcutaneously 30 min before
	3	7	+2 69	±1 04		
	6	12	+3 88	±0 94		
	12 5	7	+3 05	±0 62		
	25	7	+7 2	±1 20		Marked ataxia at 50 mgm dose
	50	7	+11 36	±0 83		
Sodium amytal	3 1	4	+0 8	±0 56		Subcutaneously 30 min before
	6 2	5	+3 91	±0 99		
	12 5	6	+6 8	±0 89		
	25	6	+9 48	±1 75		
Dilantin	25	5	+0 85	±0 73	1	Orally 75 min before
	50	5	+3 54	±2 93		
	100	6	+11 4	±2 24	1	
3 methyl 5,5 phen ylethyl hydan toin (Sandoz N₄)	12 5	4	−0 5	±1 75		Subcutaneously as 10 per cent soln in propylene glycol 45 min before
	25	5	+2 6	±0 50		
	50	12	+2 75	±1 32	1	
	100	7	+7 28	±2 64	2	
Propazone	5	7	+0 21	±0 82		Subcutaneously 30 min before
	10	6	−0 23	±0 7		
	20	6	+1 8	±0 98		
	40	6	+0 66	±0 28		
	80	6	+4 0	±1 24		
	160	6	+5 6	±1 13		
	320	5	+3 16	±1 92		
	640	5	+3 56	±1 49	4	

Sodium bromide raised the threshold to only a moderate degree, considering the large single doses administered Since this compound does not develop its full effects rapidly, it is quite probable that more pronounced actions could be demonstrated from smaller doses given repeatedly for several days Chloral hydrate had little effect, in sedative or hypnotic doses, and it was only when a

dose almost sufficient to produce complete anesthesia was administered that the threshold was significantly increased. This is in accord with the general under-

TABLE 2
Effects of depressant drugs on convulsive threshold

DRUG	DOSE PER KG		NO OF RABBITS USED	MEAN CHANGE IN THRESHOLD	STANDARD ERROR OF MEAN	NO OF RABBITS DIED	METHOD OF ADMINISTRATION
				ma	*ma.*		
Sodium bromide	0 5	gm.	5	+1.18	±1 2		Orally 2 hrs. be-
	1	gm.	7	+4 18	±0.51	2	fore
	2	gm	7	+5 93	±1 80	6	
Chloral hydrate	0 1	gm.	6	+0 41	±0 90		Orally 1 hr. before.
	0 2	gm.	6	−0 58	±0 60		Very lethargic
	0 4	gm	6	+0 28	±0 64		on 0 8 gm.
	0 8	gm	5	+10 6	±0 09	1	
Alcohol	0 5	cc.	5	+1.18	±0 91		Orally 1 hr. before.
	1	cc.	5	+1 62	±0 71		Very depressed
	2	cc	6	+4 78	±1 19		on 4 cc.
	4	cc	5	+16 28	±1 1		
Propylene glycol	4	cc	6	+2 67	±1 47		Orally 1 hr. before
	8	cc	12	+4 58	±1.23		
Paraldehyde	0 25	cc.	7	+1 43	±0 84		Orally 1 hr. before
	0 5	cc	7	+6 57	±3 60		
	0 8	cc	7	+10 85	±2 09	1	
Avertin with am-ylene hydrate	0 05	cc	6	+1 33	±0 62		Rectally 20 min. before
	0 1	cc	12	+5 12	±1 27		
	0 2	cc.	6	+8 07	±1 95		
Acetylsalicylic acid	5	mgm	5	+1 02	±0 45		Orally 1 hr. before
	10	mgm	5	+0 28	±0 92		
	20	mgm	5	+1 02	±1 44		
	40	mgm	5	−0 2	±1 62		
Acetophenetidin	0 2	gm	4	+3 0	±1 10		Orally 90 min. be-
	0 4	gm	5	+5 2	±2 27	2	fore
Acetanilid	0 1	gm	6	+1 5	±1 28		Orally 90 min. be-
	0 2	gm	5	+8 0	±2 17	1	fore
	0 4	gm	4	+8 0	±3 53	1	
Morphine sulfate	10	mgm	14	+0 63	±0 40		Subcutaneously 1
	15	mgm	13	−0 08	±0 58		to 4 hrs. before

standing of chloral action, that it is not specifically a depressant for the cortex, but acts more generally Alcohol depressed to a very marked degree, even 0.5 cc. per kilo. having a slight effect, whereas 4 cc., which caused considerable

depression and ataxia, raised the threshold 16 ma, which was the greatest average depression observed. Propylene glycol, which acts like alcohol in several respects, was much less effective on the brain. Paraldehyde raised the convulsive threshold like alcohol and propylene glycol, but was many times more powerful, considering the smaller doses required. Avertin with amylene hydrate (tribromoethanol) also produced a marked depression which was considerably greater than corresponding doses of alcohol, thus testifying to the increase in potency resulting from introduction of the bromine into the alcohol molecule.

In a somewhat special group were the coal tar analgesics acetylsalicylic acid, acetophenetidin and acetanilid. It was hoped that a depression might be demonstrated which would help to explain their known sedative and analgesic actions. With acetylsalicylic acid no change was produced with any dose. With acetophenetidin and acetanilid depression was demonstrable, but the doses required were so high compared to those clinically effective, that there can be little doubt that the therapeutically useful action does not depend on the type of change measured here.

The two large doses of morphine tried also failed to demonstrate a change in the excitability of the brain. Inasmuch as morphine exerts a unique combination of stimulation and depression throughout the central nervous system, the distribution of action, whether excitatory or depressant, seems sharply and specifically localized. In spite of apathy and considerable ataxia in these rabbits, that part of their cerebrum responsive to the convulsion stimulus was apparently not affected.

Analeptic drugs Analeptic drugs are generally used for their power to augment the reactivity of the central nervous system to reflex stimuli, or to enhance the activity of vital centers, such as the respiratory and vasomotor. In addition, some are used to lighten anesthesia and hasten the restoration of consciousness post-operatively. For the latter purpose an improvement in cerebral reactivity would seem to be almost indispensable.

However, the data in table 3 show that, at least in unanesthetised rabbits, strychnine, metrazol, coramine and caffeine had an exceedingly slight and irregular action, particularly when the high doses used are taken into account. For the area of the brain responding to the electrical stimulation used, there was no consistent lowering of the threshold of sufficient magnitude to be of practical significance. In all probability some change could have been demonstrated if the drugs had been applied locally to special cortical areas, thus avoiding effects on the remainder of the central nervous system, which here limited the tolerated dosage.

Picrotoxin differed from the other analeptics, since it caused a decrease in the threshold in the lowest doses tried and this effect was progressive until the convulsive level was reached. The 5 ma lowering of the threshold with 1.0 mgm of picrotoxin was the greatest increase in sensitivity observed in all the acute experiments. Only thyroxine surpassed it, and then only after repeated doses (table 5).

Cocaine was especially interesting because this is an excitant or convulsant drug which also has the power to paralyze nerve functions. It produced a depression of the cerebral excitability amounting to an increase in the threshold of 2.9 ma. with the 50 mgm. dose. This dose made the rabbits markedly hyperexcitable and caused a fatal convulsion in one. It is therefore probable

TABLE 3

Effects of analeptic drugs on convulsive threshold

DRUG	DOSE PER KG. SUBCUT.	NO. OF RABBITS USED	MEAN CHANGE IN THRESHOLD	STANDARD ERROR OF MEAN	NO. OF RABBITS DIED	TIME OF DRUG ADMINISTRATION
	mgm.		ma.	ma.		40 min. before
Strychnine sulfate	0.05	7	+0.2	±0.64		
	0.1	7	−1.37	±1.09		
	0.2	6	+2.07	±1.96		
	0.4	6	+0.92	±0.48		20 min. before
Metrazol	10	7	+1.05	±1.21		
	20	6	−0.58	±0.76	1	
	40	6	+0.92	±0.48		20 min. before
Coramine	12.5	6	+1.04	±1.47		
	25	5	+1.48	±0.55		
	50	4	−2.43	±0.83	1	30 min. before
Caffeine sodio-benzoate	75	6	−0.15	±1.28		
	150	6	−1.6	±0.64		30 min. before
Picrotoxin	0.125	6	−1.18	±0.81		
	0.25	6	−1.76	±0.38		
	0.5	6	−3.9	±0.86		
	1.0	6	−5.04	±1.43	3	
	2.0	6	Spontaneous convulsions			20 min. before
Cocaine hydrochloride	12.5	6	−0.4	±0.87		
	25	3	+2.16	±0.33	1	
	50	4	+2.9	±0.64		20 min. before
Mescaline sulfate	5	6	0	±1.77	1	
	10	6	+4.0	±2.50		

that the convulsive action was subcortical in origin. It is also possible that the excitant effects of cocaine may be caused by a weakening of cortical inhibition.

Mescaline was included in table 3, although in a somewhat special category, because its clinical actions are characterized by flight of ideas, hallucinations, excitation, etc. It is seen that the 10 mgm. dose raised the threshold to a possibly significant degree. This raises the question, as with cocaine, as to whether the peculiar subjective effects of this alkaloid may not arise from a depression of inhibition, thus bringing about a "dream state" comparable to that experienced between waking and sleep.

Sympathomimetic amines Because of the great interest in certain sympatho mimetic amines as central excitants (6), their effects on the convulsive threshold was studied in some detail, to obtain information which might shed light on the mechanism of their excitation

In table 4 are summarized the effects on the thresholds of benzedrine and ephedrine and their isomers, and propadrine, tyramine, paredrine, neosyneph rine, epinephrine and cobefrine These were selected from the large list of available compounds, either because of important central effects or of thera peutic importance and usage

We have previously shown in white rats that the dosage threshold for excita tion of the central nervous system by benzedrine is of the general order of 0 1 mgm per kilo (6) In rabbits, 0 6 mgm of *dl* benzedrine raised the threshold by 1 3 ma The changes, however, were not progressive with the dose, except at the 5 mgm level, where an increase of 3 1 ma was observed *d* Benzedrine was possibly more depressant than the racemic mixture, although not more than was to be anticipated from the *d* content of the mixture On the other hand, *l* benzedrine produced no consistent change in the convulsive threshold, which agreed with the previously demonstrated fact that the *d* isomer is the one which is mainly responsible for the central excitant effects of this compound

These results make it fairly clear that the restless, forced movements of benzedrine action are accompanied by depression of the brain, rather than excitation, at least according to the methods used here It would appear as though the purposeless movements were the result of the loss of cortical inhibi tion and modulation of subcortical motor drives There may be more than a fortuitous resemblance to the actions of cocaine in the benzedrine response Increase of the threshold after benzedrine was not observed by Merritt and Putnam (3) in a cat receiving a dose of 5 mgm per kilo subcutaneously, but perhaps the use of a larger number of animals and a range of dosages would reveal similar effects in this species also

Ephedrine is considerably weaker than benzedrine in producing motor ex citement and other central effects In rabbits, neither the *d* or *l* isomer pro duced any change in the electrical excitability, unless the small depression from 128 mgm per kilo of the *l* form be so regarded Propadrine is generally considered to be almost completely lacking in central excitant effects Yet in rabbits, it produced a clear cut depression equal in magnitude to that of *d*-benzedrine in the same doses This result poses the question whether the changes in cerebral excitability, by the method used, have any causal relation ship to the general excitant effects of these compounds in the central nervous system or whether they are merely coincidental

Tyramine caused practically no demonstrable changes in the thresholds after the three doses tried Paredrine raised the threshold significantly, in 2 and 4 mgm doses but failed to do so in higher doses The changes were too large to be accidental, which makes it difficult to understand why the higher doses failed With neosynephrine, epinephrine and cobefrine the results were ir regular It is possible that these compounds produced simultaneous effects

TABLE 4
Effects of sympathomimetic amines on convulsive threshold

DRUG	DOSE PER KG. SUBCUT. (mgm.)	NO. OF RABBITS USED	MEAN CHANGE IN THRESHOLD (ms.)	STANDARD ERROR OF MEAN (ms.)	NO. OF RABBITS DIED	TIME OF ADMINISTRATION
dl-benzedrine sulfate	0.3	5	−0.04	±0.14		30 min. before
	0.6	7	+1.31	±0.51		
	1.25	7	+1.6	±0.47		
	2.5	7	+0.25	±0.11		
	5	7	+3.11	±0.7		
d-benzedrine sulfate	0.19	7	−0.01	±0.78		30 min. before
	0.38	7	−0.01	±0.76		
	0.75	7	+3.4	±1.04		
	1.5	7	+2.38	±0.63		
	3	5	+1.7	±0.71	5	
	6	5	+1.56	±1.6		
	12	6	+3.38	±0.72		
l-benzedrine sulfate	0.5	6	−1.26	±0.96		30 min. before
	1	5	+1.16	±1.29		
	2	5	+0.6	±0.43		
	4	6	−2.20	±0.75		
	8	5	+1.08	±0.57	2	
	16	6	−0.8	±0.55		
	32	5	−0.98	±0.33		
	64	5	+0.16	±0.7		20 min. before
d-ephedrine hydrochloride	10	7	−0.71	±1.51		25 min. before
	20	7	−0.29	±1.49		
l-ephedrine hydrochloride	1	5	−0.58	±1.02		
	2	5	+0.32	±0.96		
	4	5	−0.18	±0.42		
	5	7	−0.45	±1.16		
	8	5	−0.12	±0.63		
	10	6	−0.1	±0.97		
	16	5	+0.28	±0.9		
	32	5	+0.22	±0.34		
	64	5	+1.88	±1.38		
	128		+1.42	±0.46		25 min. before
Propadrine hydrochloride	1	5	−0.04	±0.30		
	2	5	+1.24	±0.62		
	5	6	+3.18	±0.70		
	10	5	+2.69	±0.87	1	
	20	6	+3.46	±1.43		30 min. before
Tyramine hydrochloride	2.5	5	+0.84	±0.98		
	5	5	−1.74	±1.47	1	
	10	6	+0.16	±0.48		30 min. before
Paredrine hydrobromide	1	6	+0.21	±0.83		
	2	13	+3.20	±0.76		
	4	6	+3.68	±1.10		
	6	7	+1.34	±0.81		
	12	7	+0.12	±0.58		
	24	7	+0.67	±0.84	1	
	48		+0.66	±0.67		15 min. before
Neosynephrine hydrochloride	5	6	−0.33	±1.46		
	10	6	−2.0	±0.45	3	
	20	6	+4.0	±1.81		10 min. before
Epinephrine hydrochloride	0.5	7	+1.61	±0.60		
	1	7	−1.12	±0.53		10 min. before
Cobefrine hydrochloride	5	6	+0.4	±0.5		
	10	6	+0.8	±0.31	2	
	20	6	+3.76	±0.94		

50

on the cerebral circulation with changes in blood flow which modified any direct or intrinsic cortical changes This phase of the problem would appear to be worthy of investigation, as the results might be of value in the better under standing of epilepsy and related conditions

Thyroxine A characteristic feature of thyroid overactivity is the motor restlessness, similar to that of various other excitant drugs Since thyroid effects develop slowly, and persist for weeks, it was not feasible to use the same procedure as with the other drugs in testing the effects of different dosage levels There

TABLE 5

Effects of daily administration of thyroxin on convulsive thresholds The doses are given in mgm per kilogram body weight injected subcutaneously each day

DAY OF EXPT	NO OF RAB BITS	DRUG ADMINISTRATION PER KILO DAILY	MEAN THRESHOLD	STANDARD ERROR OF MEAN	REMARKS
			ma	ma	
1	4		22 0	±2 14	
8	9		21 6	±1 66	
15	9		22 7	±1 37	
37	9		23 6	±1 99	
76	9		24 2	±1 31	
84	9		22 7	±1 63	
107	9		25 6	±1 14	
113	10		24 0	±0 84	
120	11		23 1	±1 00	
131	11		21 6	±1 00	
Av threshold for entire control period			23 1	±0 45	
132 135	11	Thyroxin 0 5 mgm			
136	11	Thyroxin 0 5 mgm	18 8	±0 73	
137–142	11	Thyroxin 0 25 mgm			4 rabbits died
143	7	Thyroxin 0 25 mgm	17 1	±0 96	
144–149	7	Thyroxin 0 125 mgm			
150	7	Thyroxin 0 125 mgm	15 7	±1 20	2 rabbits died
151 157	5	Thyroxin 0 063 mgm			3 rabbits died
158	2	Thyroxin 0 063 mgm	16 0	±1 00	

fore, in a group of 11 rabbits the electrical convulsive thresholds were deter mined repeatedly until constant values were obtained These are summarized in table 5 where it is seen that the average threshold, without medication, was 23 1 ± 0 45 ma

On the 131st day, injections of thyroxine were begun using a dose of 0 5 mgm per kilo daily On the 136th day, the animals had lost considerable weight and the electrical excitability dropped to an average of 18 8 ma The dosage was reduced to 0 25 mgm and continued until the 143rd day During this interval 4 of the 11 rabbits died, but the survivors had a still lower threshold, i e , 17 1 ma The dosage was again halved to 0 125 mgm per kilo daily with the result

that the remarkably low average threshold of 15.7 ma. was observed on the 150th day. Five of the 7 surviving rabbits died on this and subsequent days, so that with the next lower dose of 0.063 mgm., used until the 158th day, there were only 2 rabbits to be tested, and their average threshold was 16.0 ma.

It was not possible from this series of observations to draw conclusions regarding the degree of change produced by any given level of thyroxine intake, but there was no doubt that thyroxine lowered the cerebral threshold markedly. The degree of change exhibited was much greater than that with any of the analeptic drugs. With the brain so highly reactive after thyroxine, it is not surprising that there should be impairment of poise and inadequate neuromuscular relaxation and control in clinical hyperthyroidism, assuming the latter to be etiologically comparable with the administration of thyroxine.

DISCUSSION. This study was primarily undertaken in the hope of discovering a change in electrical excitability of the brain which might shed further light on the central excitant effects of the sympathomimetic amines. Cerebral depressants and analeptics were included to afford a standard of reference for any changes produced by the amines. The results show that, in general, the depressant drugs raise the convulsive thresholds, as might be expected, but that the analeptic or excitant agents do not have a uniform type of action. Only picrotoxin showed consistently lowered thresholds. Of course the other agents may cause such great excitation of the subcortical areas that this prevented using doses which might significantly alter the functions of the cortex.

However, the sympathomimetic amines generally tended to raise the convulsive thresholds rather than to lower them, a finding similar to that with cocaine. It was suggested that their excitant effects might therefore be the result of release of subcortical levels from cortical inhibition rather than a direct excitation of the cortex. However, even this hypothesis might be difficult to sustain in view of the clear-cut depression of the excitability from propadrine, a compound which is generally considered to be practically free from central excitant effects. Obviously more extensive studies, as by ablation of specific cortical and other areas, are needed before final conclusions may be drawn as to the significance of these changes for the analeptic effects of these amines.

The demonstration of a striking increase in the excitability of the cerebrum under the influence of thyroxine fits in with the excitatory symptoms of clinical hyperthyroidism, and to that extent suggests a possible explanation of the general excitation in this condition.

SUMMARY AND CONCLUSIONS

1. An electrical device is described for measuring the convulsive threshold of unanesthetised rabbits, using a high resistance stimulator and 60 cycle alternating current. At the threshold current flow, an epileptiform convulsion is produced, which can be readily distinguished from the effects of faradizing the motor cortex. The minimal current required to produce this specific type of convulsion is approximately 20 ma. flow for 15 seconds, and is reproducible within about 1 ma., when determined repeatedly at intervals of several days.

2 Barbital compounds, dilantin, 8 methyl 6-6 phenyl-ethyl hydantoin, and propazone raised the convulsive thresholds generally proportional to the dose The barbitals showed surprisingly little difference in potency for equivalent doses Propazone appeared to be the weakest of this group

3 Marked degrees of depression of excitability were produced by the hypnotic or anesthetic group of drugs, namely, bromide, chloral, alcohol, propylene glycol, paraldehyde and avertin with amylene hydrate (tribromoethanol)

4 Of the coal tar antipyretics, acetylsalicylic acid did not change the convulsive threshold, whereas acetophenetidin and acetanilid raised it, but only in very high doses

5 Morphine, in doses of 10 and 15 mgm per kilo, did not change the threshold for the convulsive stimulus

6 The analeptic drugs strychnine, metrazol, coramine and caffeine were irregular in their effects, indicating a lack of specificity for this phase of cerebral function

7 On the other hand picrotoxin lowered the threshold to a considerable degree and in relatively low doses

8 Cocaine and mescaline raised the convulsive threshold moderately, indicating that these drugs depressed rather than stimulated cerebral function at this level It is suggested that the psychic effects or hallucinations after the use of these drugs may be due to release of cortical inhibition

9 The sympathomimetic amines generally raised the convulsive threshold to a moderate degree d Benzedrine was more effective than dl benzedrine, whereas l benzedrine was almost completely inactive, d and l ephedrine produced practically no change even in very high doses Propadrine depressed the excitability, although it is not commonly believed to have a pronounced action on the central nervous system Tyramine, paredrine and epinephrine caused only inconsistant changes but neosynephrine and cobefrine depressed the excitability in very high doses

10 The changes with the sympathomimetic amines did not agree well with their effects on the central nervous system as indicated by other methods or observations, and therefore leave some doubt as to the causal significance of the convulsive threshold changes for central stimulant effects of these amines However, this conclusion cannot be accepted without reservation until the possible role of the simultaneous circulatory changes is evaluated

11 Thyroxine lowered the threshold to epileptiform convulsions more than any of the other agents tested, the threshold being reduced from an average of 23 1 ma in the control period to 15 7 ma after 19 days of thyroxine medication This reduction suggests a possible physiological basis for the impaired neuromuscular control and poor poise of clinical hyperthyroidism

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THE MECHANISM OF ASPIRIN ANTIPYRESIS IN MONKEYS[1]

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The action of aspirin, according to clinical observation and experimental work in some species of animals, tends to follow the pattern of the organism's response to warm environments. However, for the exhibition of aspirin antipyresis the organism must be rendered febrile, although not all types of fever agents are efficient for this purpose.

Barbour (1) has shown that sensitivity to aspirin antipyresis may persist beyond the period of actual pyrexia, and employing calorimetric measurements he demonstrated that aspirin antipyresis is due essentially to an increase in heat loss, rather than a decrease in heat production. This heat loss mechanism includes peripheral vasodilation, hydremia, sweating, and polypnea.

One of us with Herrmann (2) first demonstrated that aspirin produces hydremia in fever animals, reversing the blood concentration that occurs in the onset of fever. Dontas (4) has found that the threshold for heat polypnea in normal dogs is lowered by large doses of aspirin. This mechanism of heat loss is very important in other carnivores and rodents, but in primates sweating occupies a more important place.

The present investigation concerns aspirin antipyresis in febrile monkeys controlled with normal animals with or without aspirin, in both normal and hot environments. Information was also obtained about hydremia, plasma chlorides, quantities of fluid lost by sweating, temperature and respiratory rate changes in the animals, was made.

PROCEDURES   Fourteen adult monkeys (*Macaca mulatta*) ranging in weight from 3.8 to 6.5 kilos were used   The animals successively were studied under the groups of observations shown in Table 1:

A Normal monkeys in cage with freedom of movement, room temperature around 23°C. (30 observations)

B Normal monkeys tied in supine position on padded board at room temperature of 23°C. without receiving any drug (15 observations).

C Normal monkeys in same position and environment as those in group B, but receiving 100 mgm /kgm of aspirin by stomach tube (15 observations).

D Normal monkeys tied in supine position on padded board in room at 40°C. (dry air) without receiving any drug (10 observations)

E Normal monkeys as in D, hot room, but receiving 100 mgm /kgm of aspirin by stomach tube (10 observations).

F Febrile monkeys tied in supine position on padded board in room around 23°C. without receiving any drug (10 observations)

G. Febrile monkeys in the same position and environment as those in group F, but receiving 100 mgm./kgm. of aspirin at the fever plateau (16 observations).

As a pyrogenic substance we used 5 cc./kgm. of a 25 per cent sterile yeast suspension in water, injected subcutaneously in the lateral side of the thigh. The following substances were found unsatisfactory for consistent production of fever in monkeys: Bactopeptone, B. coli vaccine, and the new Welch pyrogen. There is no simple chemical substance (e.g., cocaine, β-tetrahydro-naphthylamine) known to be satisfactory for antipyretic studies because a continuous plateau of fever is needed.

Readings of rectal temperature in the animals in the cage were made at half hour periods, and each ten minutes when the animals were tied on the padded board at the same time that the respiratory rate was counted.

Sweat was estimated quantitatively as follows: room air first was dried by drawing through an Erlenmeyer flask with sulfuric acid and a U-tube containing calcium chloride. The flow was regulated to a constant rate of 450 cc. per minute by means of a flowmeter. This apparatus was connected on one side with the vacuum and on the other with one of two exchangeable water collecting outfits H_2SO_4 and $CaCl_2$. The dry air passed through a glass plethysmograph with a rubber cuff containing the left hand of the monkey. The moisture from the skin surface went to the collecting set through an empty U-tube. The moisture collections were weighed every ten minutes. This method permits estimation of differences of 0.0001 gm. of water. In normal conditions during the ten minutes periods, the water collection ranged between 0.040 and 0.070 gm. The monkey's hand was cleaned with absorbent cotton only, avoiding the use of all cleaning fluid as mentioned by several authors, *because of differences occurring in the results. Ordinarily higher values can be* observed in the first two weights as a result of the retained moisture of preceding excretion.

After the injection of the sterile yeast suspension the animals were returned to the cage and left with freedom of movement during an approximate period of 3½ hours at which time a fever plateau of around two hours is obtained. Then the observations of fever were made with the animals again tied on the padded board.

100 mgm./kgm. of aspirin[3] were given in suspension in 25 cc. of water by stomach tube as indicated in the curves C, E, and G in figure 1. This dose appears without significant effect in normal animals, but is antipyretic for febrile animals. In the case of group E the aspirin was given upon entering the hot room; in the G group the animal received the aspirin at the onset of the fever plateau. Its effect is evident fifteen minutes after administration.

As a technique for determining the plasma specific gravity the Falling Drop method of Barbour and Hamilton was employed. Chlorides were determined according to the Van Slyke and Sendroy method.

RESULTS. *Temperature.* In thirty observations, group A, on normal monkeys, only a slight rise to 39.2°C. during the digestive period appears in the diurnal average variations with an individual maximum of 39.7°C. In the animals of group B, after tieing on the padded board, a small fall of 0.3°C. occurred in the rectal temperature when in a room at 23°C., due probably to the reduction of muscular activity and the extension of the extremities. In group C, animals tied down like the above-described controls but receiving aspirin, the change in temperature is a very small fall of 0.2°C. in an one hour period. In both groups D and E the rise of rectal temperature in response to the hot room (dry air at 40°C.) was similar whether the animals received aspirin or not; the drug appears without effect in reducing this hyperthermia. The animals of group F, after receiving the pyrogenic injection, first showed a marked fall in temperature

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[3] *Aspirin, Acetyl Salicylic Acid, Merck & Co., was employed in this experiment.*

TABLE A
Diurnal variations in cage

	TIME																					
	a m										p m											
	8 00	8 30	9 00	9 30	10 00	10 30	11 00	11 30	12 00	12 30	1 00	1 30	2 00	2 30	3 00	3 30	4 00	4 30	5 00	5 30	6 00
Temperature (°C)		39 1	39 1	39 1	39 0	39 0	39 0	38 9	38 9	39 1	38 9	39 0	39 2	39 0	39 1	39 0	39 2	39 0	39 1	39 1	39 1

TABLE B
Normal animals confined to padded cradle

	TIME (MINUTES)											
	30	60	90	120	180	240	300	330	360	390	420	450
Temperature (°C)	38 6	38 6	39 6	38 5	39 4		38 3		39 4			38 3
Respirations/min	35	34	34	32	32		30		30			29
Plasma gravity	1 0275	1 0274		1 0271	1 0272		1 0267		1 0267			1 0263
Plasma chlorides (m eq /l)	102	104		102	102		103		101			104

TABLE C
Normal animals receiving aspirin at (▲)

	TIME (MINUTES)											
	120			150				180			210	
Temperature (°C)	39 0	39 0	39 0	38 9	▲	38 9	39 9	38 7	38 7		38 7	38 7
Respirations/min	38	40	37	37	↓	37	40	40	41	42	43	43
Sweat = gms H₂O (10 min utes period)	094	071	073	0°3		060	054	053	054	049	043	045
Plasma gravity			1 0277				1 0277			1 0277		
Plasma chlorides (m eq /l)			105				104					

TABLE D
Normal animals placed in hot room at (▇)

	TIME (MINUTES)											
	120			150				180			210	
Temperature (°C)	38 6	38 5	38 3	38 2	▇	39 1	38 3	39 6	38 9	39 3	39 5	39 9
Respirations/min	35	35	35	34	↓	38	42	50	56	65	75	81
Sweat = gms H₂O (10 min utes period)	072	074	071	077			077	083	116	119	133	149	174
Plasma gravity			1 0262							1 0234		
Plasma chlorides (m eq /l)			103				102					

TABLE E
Normal animals receiving the aspirin and placed in hot room at (●)

	TIME (MINUTES)											
	120			150				180			210	
Temperature (°C)	38 5	38 5	38 5	38 5	38 4	●	38 4	38 6	39 0	39 3	39 6	40 0
Respirations/min	32	32	32	33	33	↓	35	39	45	5°°	62	71
Sweat = gms H₂O (10 min utes period)			070	064	055		060	077	114	110	155	167
Plasma gravity			1 0279							1 0°65		
Plasma chlorides (m eq /l)			100						103			

57

TABLE F

Fevered Animals Receiving Yeast Pyrogenic Injection at (×)

	30	60		80		120	180	210	300	330	360				390			410	
			×																
Temperature (°C.)	38.9 / 38.8	38.8	38.5	38.7	38.7	38.5 / 38.0	39.1	39.2	39.6	39.5	39.6	39.5	39.5	39.4	39.4	39.3	39.3	39.2	
Respirations/min.	35 / 35	36	30	35	36	34 / 36	37	39	40	40	41	44	41	41	40	40		38	
Sweat = gms H2O (10 minutes period)	.060 / .079	.059	.076	.052	.050	.046			.069	.052	.064	.060	.056	.034	.050			.042	.047
Plasma gravity	1.0285									1.0274			1.0261						
Plasma chlorides (m eq /l)	101									103			104						

TABLE G

Fevered Animals Receiving Yeast Pyrogenic Injection at (×) and Aspirin at (▲)

	30	60		80	120	180	240	300	330	360			390			410
			×							▲						
Temperature (°C.)	38.6 / 38.6	39.0	38.5	38.5 / 38.5	38.4	39.1	38.7	39.3	39.7	39.0	39.5	39.3	39.2	39.2	39.3	39.0
Respirations/min	31 / 30	30	30	30 / 30	32	36	36	39	40	40	42	40	39	39	38	37
Sweat = gms H2O (10 minutes period)	.100 / .058	.052		.074	.008			.103	.103	.105	.107	.107	.109	.100	.110	.104
Plasma gravity	1.0208								1.0254			1.0250				
Plasma chlorides (m eq /l)	103								102			101				

followed by a progressive rise until the fever peak was reached in about 3½ hours; this was held for about 1½ hours after which the temperature gradually declined.
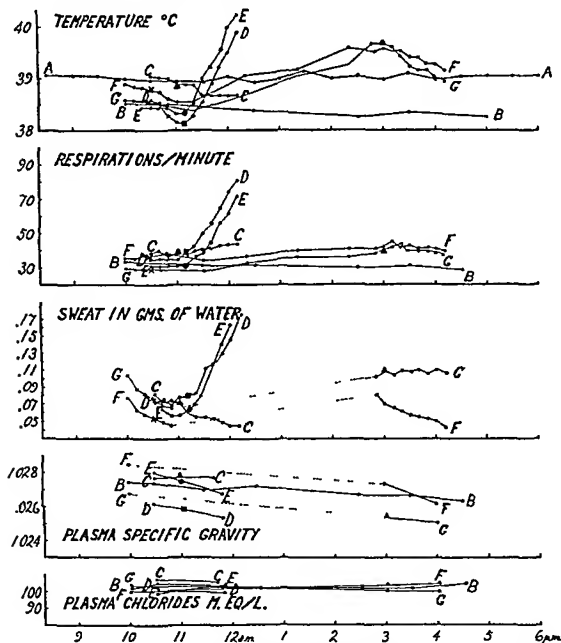
FIG 1. AVERAGE EFFECTS OF HOT ENVIRONMENTS, FEVER AND ASPIRIN ON TEMPERATURE, RESPIRATION, SWEATING, PLASMA SPECIFIC GRAVITY, AND PLASMA CHLORIDES IN MONKEYS

A Diurnal variations in cage

In the group G the fever curve was the same but when aspirin was given at the beginning of the plateau the fall in temperature was greater than those of the normal C, or untreated febrile monkeys F, and about 0.15°C. each ten minutes in the rectal temperature.

Respiration. There were no significant diurnal variations in the respiratory rates of normal monkeys, although a small fall did occur in animals after long periods of time fixed on the padded board. Group C receiving aspirin showed a very small rise in the average rate, but this was not constant.

Under the influence of the hot environment (groups D and E) there was a marked increase in the number of respirations, about 15 each ten minutes, parallel to the rise in temperature and uninfluenced by aspirin. After injection of yeast in groups F and G, a gradual rise of respirations with body temperature occurred. It is interesting to point out that aspirin did not affect respiratory rate when it caused antipyresis in febrile monkeys.

Sweat. When aspirin was given to normal monkeys the rate of water loss fell steadily from 0.084 to 0.045 gm. per 10 minutes period after two hours. This was probably due in part to moisture initially present, in part to diminution in activity and progressive fall in temperature.

In the hot room the sweat increased with the temperature and respiration, at the rate of 0.016 gm. without, and 0.018 gm. per 10 minutes period with the administration of aspirin. But in both cases the rate of water collection was doubled when the body (rectal) temperature reached 39.3°C.

In the febrile animals the rate of sweat loss became double at the maximum body temperature where no aspirin was given (group F) and then declined rapidly. On the contrary in the febrile animals of group G with aspirin, more than the double rate of excretion was maintained during the antipyresis, which therefore appears to have been due more to the sweating than to the respiratory change. The relation between plasma dilution and sweating also has been emphasized by Barbour and Brobeck (3).

Plasma specific gravity. In the control animals where seven samples of 5 cc. of blood were taken from the femoral arteries during the course of the day, the average result was a very small gradual fall in specific gravity due probably to withdrawal of blood.

Aspirin in normal animals had no effect on the specific gravity. In the hot room the usual dilution of the blood was found during the early part of the rise in temperature (at 39.3°C.) but the blood dilution was slightly more rapid with than without the aspirin. In the febrile monkeys no samples were taken during the onset of fever, but during the period of fall in temperature, either spontaneously or following aspirin, the plasma specific gravity declined. This decrease appears to be smaller under aspirin (group G) due to the large amount of water lost through sweating, and obtained from the blood.

Plasma chlorides. No significant changes have been observed during the investigation in any of the groups.

SUMMARY AND CONCLUSIONS

Yeast given aseptically by subcutaneous injection is a satisfactory pyrogenic agent for the study of antipyresis in monkeys.

In normal monkeys aspirin produces only slight decreases in temperature and appears to reduce sweat secretion.

Monkeys exhibit polypnea on exposure to hot environment, 40°C. dry air, but not as a result of yeast fever or aspirin administration either in normal or febrile condition.

Sweat accumulates more rapidly in hot environments when aspirin is given than without aspirin. This was accompanied in each case by hydremia which has a causal relation to the formation of sweat.

The amount of sweat collected is greatly increased in fever, but especially during aspirin antipyresis.

The hydremia in fever is greater in spontaneous remission than under aspirin antipyresis, evidently due to the sweating produced by the drug.

The mechanism of aspirin antipyresis in these primates is principally due to sweating, accompanied by hydremia and vasodilation.

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THE RÔLE OF THE LIVER IN THE METABOLIC DESTRUCTION OF QUININE

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The wide discrepancies which exist between the dosage of quinine administered to animals and humans and the resulting amounts which can be determined in the urine have been commented on by numerous investigators. Among these may be mentioned Merkel (1), Schmitz (2), Biberfield (3), Hartman and Zila (4), Kaiser (5), and Andrews and Webb (6). The suspicion has often been voiced that these low recoveries might be, at least in part, a result of fecal loss. But when such loss is excluded by the use of dogs with isolated intestinal loops the conclusion still holds that even 48 or 72 hours after administration of the drug only a small percentage of the absorbed quinine can be accounted for, according to data recently published by Andrews and Anderson (7). The conclusion seems inevitable that a large proportion of the drug must be changed by metabolic processes to unknown products in which the nitrogen of the quinoline and quinuclidine rings must be excreted in some form as yet unrecognized. Studies of this metabolic decomposition may involve attempts to determine the identity of these decomposition products, or to identify the site of the decomposition. The present paper presents evidence bearing on the latter and indicates the vital rôle of the liver. Studies concerning the chemistry of the metabolic decomposition are planned for later publication.

The rôle of the liver in this reaction has already been suggested by Grosser (8), Plehn (9), Porak (10), Lipkin (11), and Acton and Chopra (12). Weiss and Hatcher (13) demonstrated loss of about 18% of quinine hydrochloride on perfusion for 40 minutes through an isolated cat liver. A similar experiment continued an hour showed a loss of practically 50 per cent. It has been suggested by Bellett, Ravdin, McMillan and Morrison (14) that hepatic capillaries remove quinidine (and presumably therefore quinine) from the blood stream and hold it in loose combination, and that this action is not accompanied by destruction of the drug. However, Weiss and Hatcher (15) have expressed the opinion that the capillaries of many and all tissues are capable of holding quinine temporarily. Such claims, and also those of Giemsa (16) and Kirstner and Pantekenkoff (17) merely indicate the possibility that various organs can store quinine temporarily, a conclusion supported by the fact that quinine in common with other alkaloids is eliminated in the urine over a period of days, although the amounts eliminated after the first 48 hours are too small to affect this conclusion materially. Further, Ramsden, Lipkin and Whitely (18) and Lipkin (11) have shown that added quinine can be recovered quantitatively from a number of minced animal tissues, if the latter were previously heated, whereas from some

of these tissues, if not previously heated, recoveries were very incomplete   These results have been confirmed by us   It seems more likely therefore that some of these tissues, notably the liver contain an agent specifically responsible for the loss of quinine

If the liver is concerned with the metabolism of quinine, complete or partial hepatectomy should reflect this function by increased excretion of quinine into the urine   Complete hepatectomy, because of the abnormal condition set up, does not lend itself to such experimentation as readily as does partial hepatectomy   The latter has the advantage that it serves as an additional check on the experiment, since as indicated by Markowitz (19) the liver in dogs and rats is rapidly regenerated after such an operation   For example, when up to three fourths of a dog's liver is removed, the organ regenerates itself completely in from six to eight weeks   Thus, after this regeneration the experiment can be repeated on the same animals under what are again normal conditions

Liver damage resulting from chloroform anesthesia can be similarly produced with the same opportunity afforded to repeat the experiment on the same animal after normal conditions have been restored   Both of the above methods were used in the following experiments, partial hepatectomies having been carried out on rats and liver damage from chloroform anesthesia on a dog

EXPERIMENTAL   *Partial hepatectomy*   Five normal young adult rats were each given two preliminary periods of quinine dosage and urine collection in order to establish the normal rate of quinine excretion for each animal   The animals were so selected as to fall into two weight groups one with animals approximating 180 grams and the other 250 grams   The dosages of quinine used were roughly proportioned to their body weights (about 3 45 mgm quinine free base per 100 gram of rat) but were kept constant for all experiments regardless of variations in the weight of the animals   The quinine sulfate was adminstered in water suspension into the animal s esophagus by means of a syringe with a blunt needle and the total charge was washed in quantitatively   Each animal was fed a balanced d et of *adequate vitamin and mineral content* with water *ad libitum*   The animals were kept in cages which made possible urine collections for 3–48 hour periods after quinine dosage and the urine samples were analyzed as described by Kyker Webb and Andrews (20)

Preliminary determinations on normal urines of rats which had received no quinine showed the presence of slight amounts of some unknown substance interfering with the analytical method   For each of the rats used in this series of experiments determinations were made of the extent of this interference and the result expressed in terms of quinine per cc of urine was in all cases subtracted from the quinine values obtained later on the quinine administration experiments   The magnitude of this correction in terms of quinine varied from 2 to 4 mgm per liter of urine   As a rule the concentrations of quinine determined in the urines of the first 48 hours after quinine dosage averaged nearly 100 mgm per liter   The correction therefore was insignificant in comparison with the urinary concentrations obtained when the main mass of the quinine was being excreted

After two such administrations to the normal animals partial hepatectomies were performed and on the day following the operation the quinine dosage was repeated   The percentages of liver removed are recorded below but it should be remembered that these are only approximations obtained by comparing the actual amount of liver removed with the total weight of liver obtained from each rat when it was killed at the end of the experiment   It is possible that as indicated by Markowitz more or less liver tissue may have been formed than was present before partial hepatectomy   These figures however represent a reasonable approximation

TABLE 1

Recoveries of quinine in urine of normal rats, hepatectomized rats, rats with regenerated livers and controls

All quinine (given as the sulfate) expressed as free base. Rats 1 and 2 were males, the others females.

RAT NO.	LIVER REMOVED	QUININE FED	QUININE RECOVERED				TOTAL RECOVERY
			1st 48 hr. period	2nd 48 hr. period	3rd 48 hr. period	Total	
Normal animals—first series							
	per cent	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*per cent*
1	None	8.69	0.479	0.008	0.008	0.495	5.70
2	"	8.69	0.559	0.000	0.000	0.559	6.43
3	"	6.19	0.404	0.003	0.000	0.407	6.58
4	"	6.19	0.517	0.086	0.000	0.603	9.74
5	"	6.19	0.444	0.000	0.000	0.444	7.17
Normal animals—second series							
1	None	8.69	0.660	0.028	0.056	0.744	8.56
2	"	8.69	0.780	0.065	0.014	0.859	9.88
3	"	6.19	0.778	0.034	0.013	0.825	13.34
4	"	6.19	0.520	0.044	0.001	0.565	9.13
5	"	6.19	0.728	0.032	0.000	0.760	12.28
Partially hepatectomized animals							
1	52	8.69	1.131	0.000	0.000	1.131	13.02
2	56	8.69	1.221	0.000	0.000	1.221	14.05
3	34	6.19	1.330	0.030	0.000	1.360	21.90
4	36	6.19	1.270	0.000	0.000	1.270	20.52
5	53	6.19	1.463	0.000	0.000	1.463	23.64
Animals with regenerated liver							
1		8.69	0.609	0.026	0.004	0.639	7.35
2		8.69	0.208	0.054	0.000	0.262	3.03
3		6.19	0.566	0.033	0.028	0.627	10.13
4		6.19	0.600	0.050	0.014	0.664	10.73
5		6.19	0.914	0.024	0.022	0.960	15.67
Splenectomized animals							
2		8.69	0.700	0.000	0.000	0.700	8.06
4		6.19	0.594	0.000	0.000	0.594	9.59
Animals with an abdominal incision							
3		6.19	0.551	0.000	0.000	0.551	8.90
5		6.19	0.757	0.000	0.000	0.757	12.23

Fifty days after the date of the hepatectomies the quinine dosage was repeated for each animal at the same level as before. After the termination of this experiment, two control operations were carried out on four of the five animals previously employed in order that

the effect of partial hepatectomy on quinine excretion could be compared with that of other operative procedures and in order to rule out the effect of operative shock Two of these animals (Nos 2 and 4) had the spleen removed, the other two (Nos 3 and 5) had an abdominal incision made The following day after these operations the quinine dosage was again repeated with urine collections over 3–48 hour periods At this point all the animals were killed and the livers weighed

TABLE 2

Increase in quinine excretion in partially hepatectomized rats and in controls

RAT NO	QUININE RECOVERY NORMAL LIVER	LIVER REMOVED	QUININE RECOVERY				INCREASE IN QUININE RECOVERY AFTER HEPATECTOMY
			With hepatectomy	After liver regeneration	After splenectomy	After abdominal incision	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	5 70 8 56	52	13 02	7 35			82 6
Average	7 13						
2	6 43 9 88	56	14 05	3 03	8 06		72 2
Average	8 16						
3	6 58 13 34	34	21 90	10 13		8 90	120 0
Average	9 96						
4	9 74 9 13	36	20 52	10 73	9 59		117 4
Average	9 44						
5	7 17 12 28	53	23 64	15 67		12 23 *	143 0
Average	9 73						

The results of the whole series including the control operations, are given in detail in table 1, while recoveries of quinine are summarized on a percentage basis in table 2

The marked effect of partial hepatectomy in increasing the urinary output of quinine is obvious The increases as compared with the average of the two normal experiments, range from 72 to 143% On the other hand, neither splenectomy nor an abdominal incision caused any significant change in the level of quinine excretion This mode of experimentation therefore furnishes quantitative evidence confirming previous claims as to the vital rôle of the liver

Liver damage by chloroform anesthesia A normal male dog of 20 kg weight was freed from intestinal parasites by the use of a dose of 10 cc tetrachlorethylene and 10 cc satu

TABLE 3

Concentration of quinine (as free base) in blood and urine of a dog at intervals before and after two levels of chloroform anesthesia

Dose: 20 mg. quinine sulfate per kilo body weight

TIME OF COLLECTION	NORMAL ANIMAL			40 HRS. AFTER 30 MIN. ANESTHESIA			112 HRS. AFTER 10 MIN. ANESTHESIA			34 HRS. AFTER 1 HR. ANESTHESIA			82 HRS. AFTER 1 HR. ANESTHESIA			4 MONTHS AFTER 1 HR. ANESTHESIA		
	Blood conc.	Urine Conc.	Urine Total amt.	Blood conc.	Urine Conc.	Urine Total amt.	Blood conc.	Urine Conc.	Urine Total amt.	Blood conc.	Urine Conc.	Urine Total amt.	Blood conc.	Urine Conc.	Urine Total amt.	Blood conc.	Urine Conc.	Urine Total amt.
	mgm. per l.	*mgm. per l.*	*mgm.*	*mgm. per l.*	*mgm. per l.*	*mgm.*	*mgm. per l.*	*mgm. per l.*	*mgm.*	*mgm. per l.*	*mgm. per l.*	*mgm.*	*mgm. per l.*	*mgm. per l.*	*mgm.*	*mgm. per l.*	*mgm. per l.*	*mgm.*
0	0.78	4.5 / 4.9	0.47 / 0.25	0.77	4.8	0.16	0.78	12.25	0.14	0.71	4.8	0.52	0.86	7.5	0.42	0.74	4.9	0.21
30 min.	1.60 / 2.89	4.6 / 7.8	0.47 / 0.63	1.06	4.9	0.16	2.12	16.06	0.16	0.95	12.00	0.23	1.02	9.1	0.18	0.71	21.0	0.22
1 hr.	2.52 / 3.65	31.25 / 213.5	0.31 / 2.31	4.06	31.40	2.31	3.85	169.5	1.42	1.85	20.20	0.62	1.58	13.75	0.20	3.15	47.48	0.22
2 hrs.	3.12 / 3.53	166.0 / 106.0	3.15 / 4.99	4.55	116.8	6.89	3.66	111.4	2.84	4.2	84.20	4.62	4.31	40.30	4.39	2.60	396.5	3.80
4 hrs.	1.97 / 3.89	86.40 / 130.4	3.78 / 9.18	3.99	124.8	12.48	3.15	264.0	7.26	4.15	173.8	7.85	4.31	87.2	8.93	2.87	368.0	7.36
6 hrs.	1.46 / 1.89	85.90 / 183.0	3.22 / 5.32	4.12	125.1	8.25	3.25	47.0	4.25	3.39	146.2	7.79	3.52	131.8	6.33	2.02	321.0	8.31
12 hrs.	0.71 / 0.95	85.20 / 180.0	3.85 / 9.49	2.02	171.4	7.02	2.50	58.8	4.29	2.61	183.2	19.96	4.15	140.8	23.09	1.67	227.5	10.69
21 hrs.	1.02 / 0.87	31.80 / 53.0	2.76 / 4.60	2.61	36.2	12.67	1.06	133.8	8.45	1.69	137.7	16.52	1.40	117.1	20.81	0.97	93.1	8.05
Total quinine excreted			17.54 / 36.55			49.78			28.67			57.59			63.96			38.68

rated magnesium sulfate solution. After allowing a period of four to five days for the intestine to heal, quinine sulfate was administered by mouth in gelatin capsules at the level of 20 mgm of the sulfate per kilo of body weight. The animal was fasted for at least 12 hours before each such experiment as well as before each administration of chloroform.

The resulting concentrations of quinine in both blood and urine were followed as described by Andrews and Webb (6). Collections of both were made at periods of 30 minutes, and 1, 2, 4, 6, 12 and 24 hours after quinine administration. During all experiments the dog was kept in a metabolism cage which permitted collection of all urine voided between times of catheterization. Blank samples of both blood and urine were taken before administration of the dose. Determinations of quinine in the oxalated blood and the urine samples were made, as indicated above, by the procedure of Kyker, Webb and Andrews (20).

After two such experiments were run the animal was put under chloroform anesthesia for 30 minutes. Forty hours afterwards, quinine sulfate was administered and blood and urine levels were followed as before. The same experiment was also carried out 112 hours

TABLE 4

Increase in percentage of urinary quinine excretion in a dog with liver damaged by chloroform anesthesia

PERIOD OF CHLOROFORM ANESTHESIA	TIME AFTER ANESTHESIA	QUININE RECOVERY	INCREASE IN QUININE RECOVERY
		per cent	*per cent*
None		7 9	
30 min	40 hrs	13 7	73
	112 hrs	7 9	0
60 min	34 hrs	15 7	99
	82 hrs	17 4	120
	4 months	8 9	13

after the time of anesthesia. Following this the animal was subjected to chloroform anesthesia for one hour and two quinine administration experiments 34 and 82 hours afterwards were run. Four months afterwards when normal liver conditions had been established, a last dose of quinine was administered, followed by the same determinations as before.

The data resulting from this series of quinine administration experiments are presented in table 3 in terms of concentrations and total amounts of quinine in blood and urine. The figures listed for the normal animal are those which resulted from both experiments. Since the animal gained somewhat in weight during the total course of the experiment (about six months), the dosages of quinine sulfate, based upon 20 mgm per kilo, increased proportionally. Hence, as in the case of the partial hepatectomies on rats, the summarization in terms of percentage excretion shown in table 4 is the more significant. Since the two experiments on the normal animal produced total urinary recoveries of 5 2 and 10 6% of the dose respectively, the percentage recoveries of table 4 are calculated on the average of these two figures, or 7 9 per cent. The increase resulting from liver damage, particularly in the case of the longer period of anesthesia, is quite evident. This finding is in agreement with the higher concentrations of quinine maintained in the blood stream for several hours after its administration when

partial liver disfunction has been produced. On the other hand, the urinary excretion of quinine 112 hours after 30 minutes of anesthesia shows no increase. Such a finding is not unexpected. Although the experiments of Rosenthal and Bourne (21) indicate some impairment of liver function for 8 days after one-half hour of chloroform anesthesia, the effect after three or four days is comparatively small. It would appear therefore that a period of 112 hours after one-half hour of such anesthesia suffices for the establishing of normal conditions, at least as far as quinine destruction is concerned. The exact duplication of our average normal figure for urinary excretion (7.9 per cent) at this time is, of course, purely fortuitous. More extensive damage, resulting from one hour of chloroform anesthesia, would necessitate a longer period for recovery but the data indicate that after four months normal conditions had been reestablished. We thus confirm by different technique the claims of some of the earlier investigators as to the rôle of the liver in quinine destruction. Further studies concerning the isolation of the active agent from liver tissue are in progress and will be reported later.

It is of interest in this connection that the excretion of quinine, after standard dosage, has recently been proposed by Miano (22) as a test for liver function.

CONCLUSIONS

1. The removal of from one-third to one-half of the liver of rats causes an increase in urinary excretion of quinine of from 72 to 143 per cent over that observed with the same intact animals. After a period of four weeks, allowed for regeneration of the liver tissue, the excretion again falls to normal levels.

2. Splenectomy or such surgical procedures as an abdominal incision does not interfere with the normal rate of excretion.

3. Damage to the liver of a dog due to chloroform anesthesia results in maintenance of higher levels of quinine in the blood stream for a longer period of time than normal and also raises the percentage of urinary excretion. A longer period of chloroform anesthesia increases this effect. After a period of four months for the reestablishing of normal conditions, the blood levels and urinary excretion following standard quinine dosage in the same dog, were found to have returned to normal.

4. Our results indicate the presence of some agent in the liver which is evidently active in the metabolic destruction of quinine.

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STUDIES ON ANTIMALARIAL DRUGS

The Metabolism of Quinine in Pregnant Animals[1]

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The use of quinine during pregnancy, either for the initiation of uterine contractions, or for the control of malaria, is not without danger to the fetus. There are several reports dealing with the toxic effects of quinine administered to induce labor (1–4). In those experiments the amount of quinine found in the fetus after such treatment was not determined because of the lack of a sufficiently sensitive and specific method. Investigations dealing with the placental transmission of quinine have been made on the human being (4, 5), rabbit (4), dog (5), and guinea pig (6). Although these experiments were chiefly qualitative, they demonstrated the ability of the placenta to transmit quinine to the fetus. In two instances, attempts were made to determine quantitatively the placental transmission of quinine (4, 5). In the human being, quinine passes through the placenta in the middle of the second trimester (5), but a quantitative determination of the capacity of the placenta to transmit quinine, in relation to the stage of pregnancy is lacking.

EXPERIMENTAL. The experiments reported here were designed to show the influence of pregnancy on the metabolism of quinine both in the maternal rabbit and in the fetus. The stage of pregnancy was accurately known from the mating date. In four rabbits, pregnancy was prolonged by the intravenous administration of 10 units of Gonadogen (Upjohn) on the 25th day of pregnancy. Quinine was given intravenously as a 1% solution of the hydrochloride in water. The dosages and analytical data are expressed in terms of the free alkaloid. In all experiments except those in which quinine was given by mouth, 10 mgm./kg. of quinine was given intravenously. The animal was sacrificed one hour later and samples taken for the determination of the quinine concentration. In one group of experiments, the time interval was varied.

Three fetuses of each litter were analysed separately except for the 12, 13 and 14 day stages in which all of the fetuses were combined for analysis. Earlier stages were too small for analysis. The organs from several fetuses were usually combined. In each case, three placenta were analysed, one intact and the other two separated into maternal and fetal portions. The maternal tissues were dissected as rapidly as possible, weighed and an aliquot dissolved in 2% NaOH. The quinine content was determined by the method of Kelsey and Geiling (7).

RESULTS   *Quinine in the fetus*   The total quinine in the fetus determined one hour after administration to the maternal animal increased rapidly as pregnancy advanced, tending to parallel the increase in fetal weight   The concentration of quinine in the fetus was at the highest level on the 12 day (fig 1), slowly dropped from the 12th to the 23rd day, and gradually increased from then until term   The concentration showed a marked fall in the post term fetuses

The concentration of quinine in tissues of fetuses older than 23 days was determined one hour after administration to the maternal animal   The liver ranged from 0 3 to 0 9, the kidney from 0 5 to 1 9 and the lung from 0 8 to 5 8 micrograms per gram   The quinine content of a full term fetus was found to be 4 6 micrograms two hours after a single oral dose of 20 mgm /kg   After repeated daily oral doses of 20 mgm /kg from the 21st day of pregnancy, a
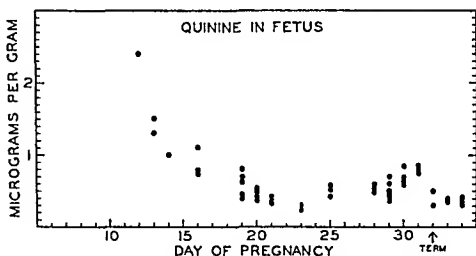
FIG 1   QUININE CONCENTRATION IN THE FETUS ONE HOUR AFTER INTRAVENOUS ADMINISTRATION OF 10 MGM OF QUININE PER KILOGRAM TO THE MOTHER

Points from the fifteenth day to the thirty fourth day represent individual fetuses   the others represent the average of each litter

full term fetus contained 8 9 micrograms of quinine two hours after the last dose In another group of animals the quinine was found to have practically disappeared from both the maternal and fetal tissues 12 hours after the injection of 10 mgm /kg   In four hours the concentrations were less than 10% of those observed 10 minutes after the injection

The fetal portion of the placenta always contained more quinine than the maternal portion   The average of 15 fetal placentas was 6 9 micrograms total quinine, or a concentration of 3 1 micrograms per gram   For 15 maternal placentas the average values were 2 0 and 1 1 respectively

Quinine in the maternal tissues   The relationship between the stage of pregnancy and the concentration of quinine in the tissues of the maternal animal one hour after injection are shown in figs 2 and 3   Most of the tissues show the lowest values at the end of the second trimester, with rising values toward term   This is especially marked in the liver, spleen, blood and lung   The tissue levels

observed in these experiments were always very low compared to levels found in similar experiments with dogs, cats and guinea pigs. The discrepancy was especially noticeable when values for liver quinine were compared. These
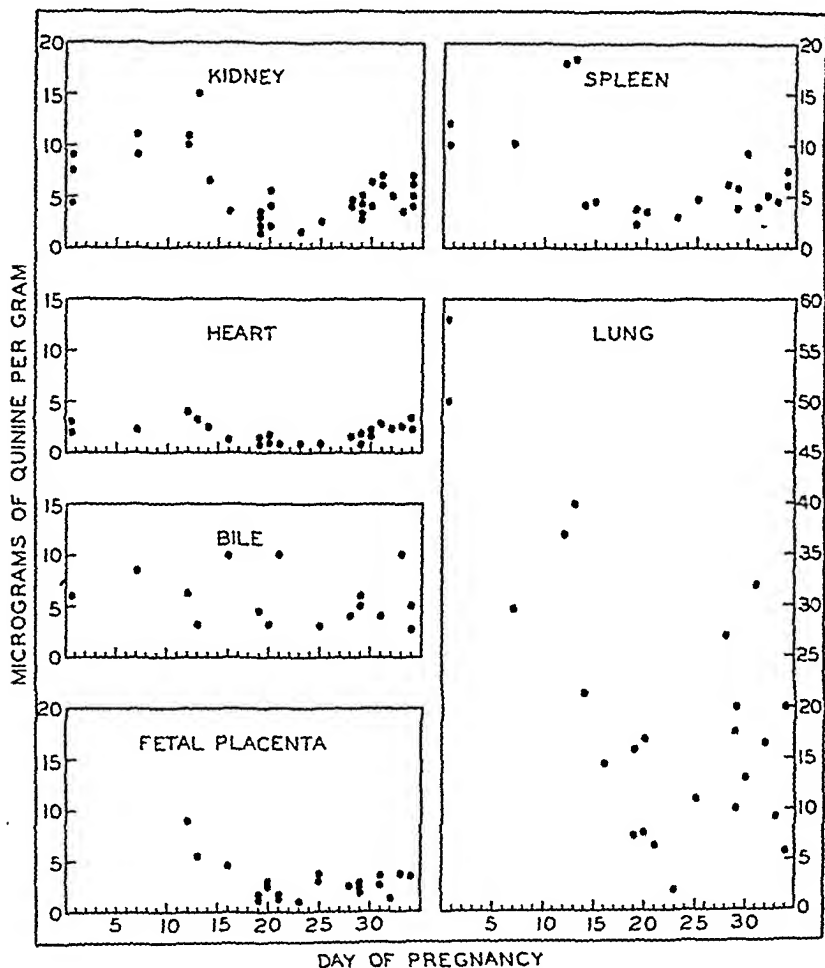

FIG. 2. QUININE CONCENTRATION IN MATERNAL ORGANS, BILE, AND FETAL PLACENTA OF THE RABBIT ONE HOUR AFTER THE INTRAVENOUS INJECTION OF 10 MGM. PER KILOGRAM OF QUININE TO THE MOTHER

observations led to a study of the ability of the rabbit liver to destroy quinine *in vitro*, which will be reported in detail elsewhere (8, 9). These studies have shown the rabbit liver to be much more effective in destroying quinine than the liver of the cat, dog, guinea pig and several other species.

DISCUSSION. When standard experimental conditions are observed with respect to dosage, mode of administration, and the time interval following the administration of the quinine, there is a progressive increase in the quantity of
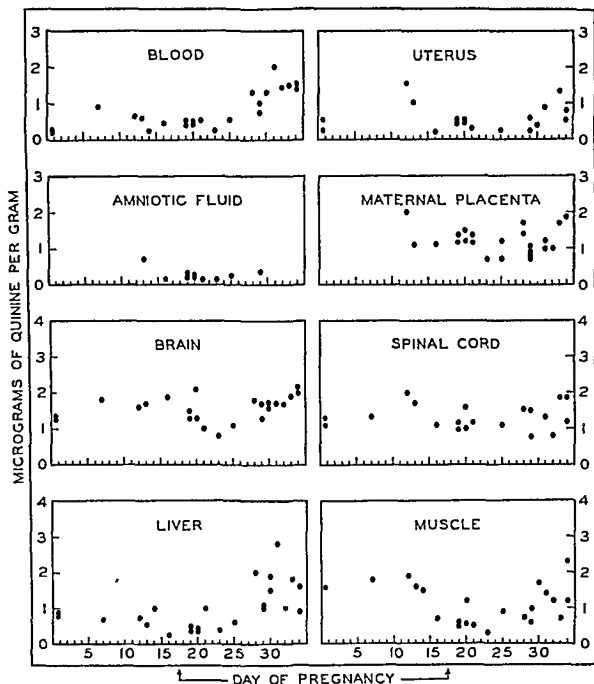
FIG 3 QUININE CONCENTRATION IN MATERNAL ORGANS OF THE RABBIT ONE HOUR AFTER THE INTRAVENOUS ADMINISTRATION OF 10 MGM PER KILOGRAM TO THE MOTHER

quinine passing from the maternal animal to the fetus as pregnancy progresses This was found to be true for arsenic by Snyder and Speert (10), and for other substances (11, 12). In our experiments, prolonging pregnancy resulted in less quinine in the fetus than at term. Under similar conditions Snyder and Speert

(10) found more arsenic in the fetus after term. The fall in quinine concentration in the post-term fetus may be due to a decreased rate of transmission, to an increased destruction in the fetus or to excretion by the fetus to the maternal animal. Experiments *in vitro* suggest an increased destruction by fetal livers (8), while we have found that the placenta is capable of transmitting quinine from the fetus. Quinine was detected in maternal tissues shortly after its injection into the fetus.

At term the greatest amount of quinine was found in the fetus *10* minutes after injection into the maternal animals. Progressively smaller amounts were found at later intervals. This is in contrast with the results obtained with arsenic by Snyder and Speert (10), who found that the amount of arsenic traversing the placenta was directly proportional to the time at which the animal was sacrificed after the injection.

There is a considerable difference in the behavior of the maternal and fetal portions of the placenta toward quinine. The amount of quinine passing through the maternal placenta was twice as great as that passing through the fetal placenta in a ten minute period. The fetal placenta, although affording ready passage to considerable quantities of quinine, does have the ability to hold back or store within itself relatively large amounts of the alkaloid for a short period of time, thus affording some protection to the fetus against the alkaloid. Quinine is said to be fixed readily by the capillaries and held very loosely (13). This may explain the retention of large amounts by the fetal placenta which has an abundance of capillaries. The trophoblastic cells of the placenta, which have marked phagocytic power (14), may aid in this rapid fixation and transmission to the fetus. On the other hand arsenic, being a capillary poison, may be able to pass the placental barrier only after injury to the capillaries. Arsenic is firmly fixed by the tissues and excreted very slowly (15, 16).

The high concentration of quinine in the rabbit fetus in the early stages of pregnancy is difficult to explain. At this stage the fetal blood and the maternal blood are widely separated. In the final stage of pregnancy, the maternal blood is separated from the fetal blood only by an endothelial layer of cells (17). However, on the 12th day the tissue separating the two blood streams is fetal endothelium, strands of mesenchyme in places, and the plasmodium, which is often extremely thin. In addition, the fetal circulation is well established. The fetal vessels extend almost to the tips of the ingrowing trophoblastic folds and villi. Polymorphonuclear leucocytes are abundant in the venous plexus, vacuoles, and syncytium where these have been filled with blood. At this stage of pregnancy, the leucocytes and trophoblastic cells must be considered as playing a possible rôle in the transmission of the quinine.

After intravenous injection quinine was found in the highest concentration in the maternal lung, kidney and spleen, with the heart, bile, liver, muscle, brain, spinal cord, blood and uterus next in order. This organ distribution agrees in general with that reported by Lipkin (18). In the rabbit the lung initially fixed quinine in the greatest quantities. Lipkin reported only two analyses on rabbit

lungs and did not find the quinine content in them particularly high as compared with other organs Hatcher and Weiss (13), studying quinine distribution in the cat, found that quinine is rapidly fixed by the capillaries, and that the lung, liver, kidney, and brain contained quinine in higher concentration than muscle or blood

On the basis of quinine concentration, the ability of the liver to detoxify quinine is evidently greater in the non pregnant animal and in the first and second trimesters of pregnancy than in the third trimester. In spite of the fact that the concentration of quinine rises to a higher level in the liver during the third trimester than at any time during the first two trimesters, the concentration of quinine did not increase as much in other organs as would be expected if the liver was the sole source of the enzymes responsible for the destruction of quinine.

The changes in the rate of quinine detoxication in the rabbit during pregnancy is of practical significance That similar changes occur in the human is suggested by the work of Abe (19) on placental enzyme activity In the human, toxic symptoms are less likely to occur during the second trimester This is in accord with the increased detoxifying power occurring in the second trimester as seen in the rabbit with respect to quinine.

SUMMARY

Quinine readily passes the placenta of the rabbit and may be found in the fetus in highest concentration on the 12th day of pregnancy It is rapidly destroyed or excreted and repeated daily doses show little tendency to accumulate in the fetus or maternal organs The relatively high concentration in the fetal portion of the placenta suggests that some degree of protection is afforded by this organ

The rabbit has a remarkable ability to destroy quinine, which is reflected by the very low concentrations found in the tissues shortly after intravenous injection The stage of pregnancy has a distinct effect on the ability of the rabbit to destroy quinine At the end of the second trimester, quinine is most rapidly destroyed, but at term there is more quinine stored in the tissues than in a non pregnant animal

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STUDIES ON ANTIMALARIAL DRUGS

The Distribution of Quinine Oxidase in Animal Tissues[1]

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The ability of certain tissues to destroy quinine *in vitro* has long been recognised Plehn (1), Grosser (2), Lipkin (3) and Weiss and Hatcher (4) perfused the livers of cats, dogs and sheep with a quinine containing perfusate and obtained destruction of as much as 75% of the added quinine, while Ramsden, Lipkin and Whitley (5), Lipkin (3) and Anderson and Andrews (6) showed destruction of the alkaloid on incubation with various minced tissues Since it is by no means certain whether the efficacy of quinine in malaria is due to the quinine *per se* or to some metabolic product, the nature of this *in vitro* breakdown of quinine is of considerable importance We have examined the problem in some detail during the past year and present in this report our quantitative studies on the quinine oxidase in the tissues of various animals

MATERIAL AND METHODS The various species of animals used were sacrificed without anesthesia the tissues were removed blended with four volumes of Ringer Locke solution and strained through several thicknesses of gauze[2] Aliquots of 1 0 5 0 and 10 0 cc representing 0 2 1 0 and 2 0 grams of tissue respectively were added to beakers containing 0 1 mgm of quinine as the hydrochloride in 10 cc of Ringer Locke solution The total volume was adjusted to 20 cc by the addition of Ringer Locke After incubation for one six or twelve hours at 37° the samples were analyzed for residual quinine by the method of Kelsey and Geiling (7) In all cases the recovery from unincubated samples was within 5%

RESULTS AND DISCUSSION The results can be considered in two parts first, a comparison of the amounts of quinine oxidase in the liver of various species, and secondly, the distribution of the enzyme in tissues other than the liver

The per cent destruction of 0 1 mgm of quinine by the liver of various species is presented in tables 1 and 2 Since the rabbit liver is so much more active, smaller aliquots had to be used and the results are therefore presented separately Next to the rabbit, in descending order of activity are the rat mouse, cat and guinea pig, with the rat having about 50% the activity of the rabbit The livers of the

[3] This extract can be stored at room temperature for at least 6 hours without loss of activity Cell free NaCl filtrates of liver were found to contain appreciable amounts of the enzyme Dehydration of the filtrate *in vacuo* resulted in a light tan powder which could be resuspended in water with considerable activity remaining The activity of the powder rapidly decreased on storage Since oxygen is essential for the reaction and since preliminary studies suggest the end product of the reaction to be an oxidation product the enzyme is referred to as quinine oxidase[3]

77

monkey, pig, steer, dog, chicken and frog show little or no activity under the conditions of these experiments. A small but definite conversion occurred with fresh human liver. The amount of liver available was insufficient for quantitative analysis.

The data obtained for the activity of enzymatic tissues other than the liver are presented in tables 3 and 4. From Table 3, it can be seen that many of the

TABLE 1

The quinine oxidase activity of the livers of various species expressed as per cent destruction of 0.1 mgm. of quinine and added to each after one or six hours' incubation at 37°C. using 0.2, 1.0 and 2.0 grams of tissues

SPECIES	HRS. INCUBATION	0.2 GMS.	1.0 GMS.	2.0 GMS.
Rat	1*	50	95	95
	6	87	97	98
Mouse	1	10	42	55
	6	19	88	91
Cat	1	26	41	17
	6	23	63	15
Guinea pig	1*	0	10	12
	6*	8	35	45
Monkey	6	9	18	17
Pig	1	0	20	13
	6	8	20	13
Sheep	6	0	0	10
Steer	1	0	0	9
	6	0	8	18
Dog	6	0	0	0
Chicken	6	0	0	0
Frog	6	0	10	7

* Average of two experiments.

TABLE 2

The quinine oxidase activity of the liver of the rabbit expressed as per cent destruction of 0.1 mgm. quinine after one, six and twelve hours incubation at 37°C., using 0.02, 0.04, 0.06, 0.08 and 0.1 grams of tissue

HOURS INCUBATION	0.02 GMS.	0.04 GMS.	0.06 GMS.	0 08 GMS.	0.1 GMS.
1	18	37	51	63	78
6	54	82	95	98	100
12	69	94	100	100	100

rabbit tissues have the ability to convert quinine, though none to the extent of the liver. The lungs, kidneys, uterus and heart are the most active, followed by the leg muscle, small intestine, spleen and adrenals. No activity could be demonstrated in the brain, large intestine and fetal and maternal placentas. Whole blood showed only slight activity, 2.0 grams converting about 15% of the added quinine.

In the other species examined, slight activity was seen in the lungs and kidneys of the rat and guinea-pig, in the lungs of the mouse, and in the kidney of the

TABLE 3

The quinine oxidase activity of tissues of the rabbit expressed as per cent destruction of 0 1 mgm of quinine after one and six hours incubation at 37°C   No activity could be demonstrated in the brain, large gut and fetal and maternal placentae   The blood showed only a slight tendency to split quinine

ORGAN	HRS INCUBATION	0.2 GMS	1 0 GM	2 0 GMS
Lung	1*	13	59	64
	6	38	97	97
Kidney	1*	5	35	45
	6	16	80	84
Uterus (non pregnant)	1	9	24	46
	6	13	78	96
Uterus (pregnant)	1	11	54	75
	6	22	85	95
Heart	1	0	18	36
	6	5	50	68
Leg muscle	6	9	25	34
Small Intestine	1*	0	20	20
	6	0	25	37
Spleen	1*	7	24	27
	6*	16	48	48
Adrenals	1*	5	20	20
	6	20	46	51

* Average of two or more experiments

TABLE 4

The quinine oxidase activity in the tissues of various species   The figures denote the per cent destruction of 0 1 mgm of quinine after 6 hours incubation at 37°C with 2 grams of tissue

SPECIES	LUNG	KIDNEY	SMALL INTESTINE	BRAIN
Rat	18	18	0	0
Mouse	15	0	0	0
Guinea pig	12	20	0	
Dog	0	0	0	
Cat	0	0	0	
Monkey		0		
Chicken	0	30	0	0

TABLE 5

The amount of quinine in mgm /kg one hour after the intravenous injection of 10 mgm /kg of quinine as the hydrochloride

SPECIES	LIVER	LUNG	KIDNEY	HEART	SPLEEN	MUSCLE	BILE
Rabbit (normal female)	0 9	66 6	4 8	2 5	12 1	1 5	6 2
Rabbit (at term)	2 8	32 2	6 7	2 5	3 9	1 4	4 0
Dog (normal female)	23 1	34 2	12 4	7 8	32	3 8	
Dog (at term)	8 3	39 5	15 8	8 7	28	3 6	10 1
Chicken (av of 5 birds)	22	13	17	10	21	5	29

chicken. Small intestine and brain showed no activity. These results are somewhat at variance with those of Lipkin (3) and of Anderson and Andrews (6). Anderson and Andrews reported quinine-splitting activity in unstated amounts in the brain, intestinal wall and kidneys of mice while Lipkin obtained 66.76% destruction of 12.3 mgm. of quinine by the small intestine of the guinea-pig and complete destruction of 4.6 mgm. of quinine by the large intestine of the rabbit.

That the rate of disappearance of quinine injected into the living animal is related to the amount of quinine oxidase of the tissues is suggested by the data presented in table 5. In each case the animal received 10 mgm./kg. intravenously of quinine as the hydrochloride, and one hour later the tissues were analysed for quinine. With the exception of the lung, the concentration in the tissues of the rabbit was in every instance lower than that in the corresponding tissues of the dog and chicken. This is most striking in the case of the liver which in the rabbit contained only very small amounts of quinine, suggesting a high rate of destruction. Support for this view comes from the data for the quinine content of the bile, which is appreciably higher in the chicken and dog than in the rabbit, indicating that in the case of the first two, more quinine is excreted in unchanged form.

SUMMARY AND CONCLUSIONS

The quantitative distribution of the quinine oxidase has been studied in tissues of various animals. The rabbit contains the greatest amount of the enzyme of all species studied, the liver being especially rich. The rat liver shows about 50% of the activity of rabbit liver and the mouse somewhat less. Cat and guinea-pig livers show appreciable amounts but those of the monkey, pig, steer, chicken, dog and frog show little or no activity. Some activity was apparent in human liver.

Most other tissues of the rabbit contain the enzyme, the lungs, kidney and uterus being especially active. No activity could be demonstrated in the brain, large intestine, and fetal and maternal placentas. The blood showed slight activity.

In the other species studied, the lungs of the rat, mouse and guinea-pig, and the kidney of the rat, guinea-pig and chicken showed slight activity.

The rate of disappearance of injected quinine is probably determined to some extent by the amount of quinine oxidase present.

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STUDIES ON ANTIMALARIAL DRUGS

The Influence of Pregnancy on the Quinine Oxidase of Rabbit Liver[1]

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In the preceding paper, we have described the relative ability of tissues of various species to destroy quinine *in vitro* (1). The rabbit showed the greatest activity in this respect, the liver being an especially rich source of quinine oxidase. Furthermore, evidence was presented to show that the rate of disappearance of injected quinine is probably determined to some extent by the amount of enzyme present

Recent studies by Burton and Kelsey (2) have shown that when quinine is injected intravenously into rabbits during various stages of pregnancy, the concentration in the maternal liver increases as term approaches. The following studies were undertaken to see whether the activity of quinine oxidase *in vitro* was affected by pregnancy, and to determine the time of first appearance of this enzyme in the young.

MATERIAL AND METHODS Seventeen rabbits ranging from 20 days of pregnancy to 42 days post partum and 9 non-pregnant and non nursing controls were used in these experiments The animals were sacrificed without anesthesia, the liver removed, weighed, blended in a Waring blender with 4 volumes of Ringer-Locke solution and filtered through several thicknesses of gauze Aliquots ranging from 0 5 to 5 0 cc (0 1–1 0 gm liver), were added to 100 cc beakers containing 10 cc of a quinine HCl Ringer-Locke solution representing 1 mgm of quinine as the free base together with sufficiently more Ringer-Locke to make a total volume of 20 cc The samples were rotated to ensure mixing and incubated at 37°C for one hour and then analysed for quinine by the micromethod of Kelsey and Geiling (3) All samples were analysed in duplicate In all cases, recovery from unincubated samples was within 5%

To afford a basis of comparison, a standard reference curve was prepared as follows Approximately 10 grams of liver were taken from each of 8 non pregnant rabbits and the total blended with 4 volumes of Ringer-Locke solution and filtered through gauze Closely spaced aliquots ranging from 0 1 to 3 0 cc were incubated as before with 1 mgm quinine samples and the percentage destruction of quinine determined in each This was plotted against the amount of tissue in grams The resulting curve is illustrated in fig 1 In order to express the activity of the various livers in terms of the standard, amounts giving between 20% and 80% destruction were expressed as percent of the amounts of pooled liver giving a similar destruction The several values for each animal were then averaged and the average deviation determined In no case did this exceed 10%

In determining the first appearance of the enzyme, we removed livers from the fetuses or the young of the various mothers In the case of very small animals, the livers of the entire

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litter were pooled. The activity was determined as with the maternal livers except that in some cases only 0.1 mgm. of quinine was used. The symbols 0, ± and + indicate that 1 gram samples gave a destruction of less than 5%; between 5 and 13%; and between 13 and 20% respectively.

RESULTS AND DISCUSSION. The results of these experiments show a very distinct drop in the enzymatic destruction of quinine during late pregnancy and during the early post-partum period (table 1). At 20 days pregnancy one rabbit showed a considerable fall in activity while two showed normal activity. At 26
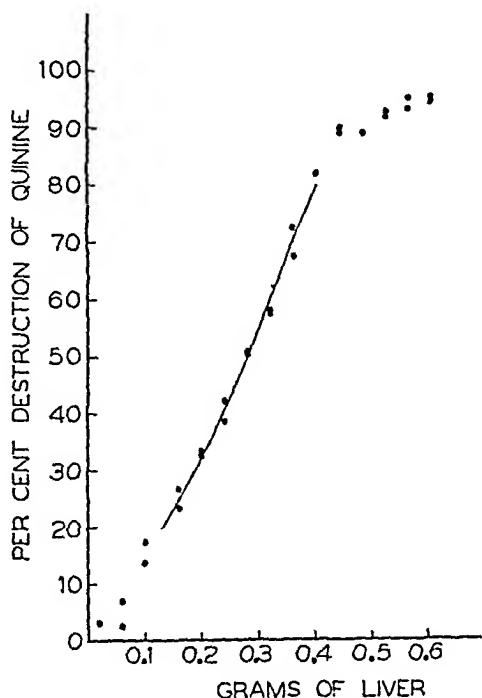
FIG. 1. THE RELATIONSHIP BETWEEN THE AMOUNT OF LIVER IN GRAMS AND THE PER CENT DESTRUCTION OF 1.0 MGM. OF QUININE IN A TOTAL VOLUME OF 20 Cc. AFTER 1 HOUR INCUBATION AT 37°

days, all rabbits examined showed less than 50% activity, and at 29 days and term less than 25% activity was found. The rabbits examined one and two days after delivery are not in good agreement: in both cases only one of each pair was low in activity. Activity was still low in at least one of the animals 16 days post-partum and at 32 days post-partum. At 42 days, the normal activity was restored.

The lessened activity during late pregnancy is apparently due to a reduced amount of enzyme rather than to the presence of an inhibitory substance.

Evidence for this was obtained by studying the activity of a mixture of equal parts of the liver suspensions from rabbits no 9 and no 16   Subsequent analyses

TABLE 1

The quinine destroying activity of non pregnant pregnant, and post partum rabbits, expressed as the per cent activity of the standard preparation (see fig 1)

RABBIT NO	DAYS	PER CENT ACTIVITY	
		Maternal animal	Offspring
Non pregnant			
1		94	
2		75	
3		100	
4		91	
5		132	
6		152	
7		121	
8		100	
9		94	
Pregnant			
10	20	104	0
11	20	32	+*
12	20	113	+*
13	26	38	0
14	26	43	0
15	26	48	
16	29	22	+*
17	at term	19	±
18	at term	22	±
Post partum			
19	1	72	
20	1	21	±
21	2	73	30
22	2	16	±
23	16	41	42
24	16	71	13
25	34	50	55
26	42	110	39
			48
			130
			47

* Using 0 1 mgm of quinine

showed that the mixture had an activity of 63% which is the activity to be expected from such a mixture providing no inhibition occurs

In regard to the appearance of quinine oxidase in the offspring, little or no activity was present before birth   In one of each of the 2 and 16 day young, and

in all older litters, appreciable activity was present though even by the 42nd day after birth it had not, except in one instance, reached the activity of the non-pregnant adult. The enzyme apparently appears shortly after birth but its maximum efficiency has not been attained until after the animals are weaned.

The reduced ability of the rabbit liver to destroy quinine *in vitro* during the later days of pregnancy agree with the findings of Burton and Kelsey (1). These investigators administered quinine to rabbits throughout pregnancy and an-alysed the tissues to study its distribution. They found that during the third trimester, the liver of the mother contained appreciably more quinine than during the earlier stages or than in the liver of non-pregnant animals. In view of our findings regarding enzyme activity, this increased accumulation of quinine during late pregnancy may be due to a decrease in enzymatic destruction of the drug.

The regularity with which this enzyme can be demonstrated in the rabbit liver contrasts with the findings of various investigators (see Bernheim and Bernheim (4)) on the esterase splitting atropine, which is found in only approximately one rabbit out of three. The non-pregnant control animals (table 1) show a rather surprisingly similar activity especially when one considers that the animals were not all of the same strain and the experiments were done throughout the year. In addition, we have examined a number of male animals and have found that these also exhibit an activity approximating that of the standard preparation. Care was exercised to use only healthy animals since in two animals showing severe parasitic infections of the liver, one with chronic hepatitis due to round worm infestation, the other with adenomatous proliferation of the bile ducts due to coccidiosis, the quinine oxidase activity was 37% and 69% respectively that of the standard preparation.

SUMMARY

The ability of rabbit liver to oxidize quinine was studied in normal animals and in animals in various stages of pregnancy. In normal animals, the enzyme is present in rather constant amounts but becomes considerably reduced during late pregnancy and during the early post-partum period. This difference appears to be due to a reduction in the amount of enzyme present rather than to the presence of an inhibitory substance.

The enzyme can be demonstrated in young animals shortly after birth but is not found in amounts comparable to that of the normal adult until after the weaning period.

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THE RELATION OF MOLECULAR CONFIGURATION TO THE RATE OF DEAMINATION OF SYMPATHOMIMETIC AMINES[1] BY AMINASE

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We recently reported a systematic study and interpretation of the relation of molecular structure to the rate of oxidation of phenolic sympathomimetic amines by phenolase (1) That such a relationship did exist and was readily interpreted on a physical chemical basis encouraged us to determine whether this same approach could be extended to another enzymic reaction

The purpose of this research has been, then, to determine whether there existed a systematic relationship of structure to rate of deamination of sympathomimetic amines in the presence of aminase[3] We have also offered interpretations of our observations, wherever possible

PROCEDURE Twenty two sympathomimetic amines were studied in the course of this research They were

1 β phenylethylamine
2 β methylphenylethylamine
3 β,β dimethylphenylethylamine
4 β phenylethylmethylamine
5 β methylphenylethylmethylamine
6 β β dimethylphenylethylmethylamine
7 β phenylethyldimethylamine
8 β methylphenylethyldimethylamine
9 β β dimethylphenylethyldimethylamine
10 γ phenylpropylamine
11 β phenyl β hydroxyethylamine
12 β(4 hydroxyphenyl) ethylamine (tyramine)
13 β (4 hydroxyphenyl) ethylmethylamine
14 β(4 hydroxyphenyl) ethyldimethylamine (hordenine)
15 β(4 hydroxyphenyl) β hydroxyethylmethylamine (synephrine)
16 β(3 hydroxyphenyl) β hydroxyethylmethylamine (neosynephrine)
17 β(2 hydroxyphenyl) ethylamine
18 β(2 hydroxyphenyl) ethylmethylamine
19 β(3,4 dihydroxyphenyl) ethylamine
20 β(3,4 dihydroxyphenyl) ethylmethylamine
21 β(3,4 dihydroxyphenyl) β hydroxyethylmethylamine (adrenalin)
22 β(3 4 dihydroxyphenyl) β ketoethylmethylamine (adrenalin ketone)

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[1] The term sympathomimetic amine has been used in this paper to include compounds having a basic phenylethylamine structure (except in one instance) and in general, but not invariably, possessing pressor properties

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[3] Not included in this report are any compounds having an aliphatic isopropylamine side chain such as propadrine ephedrine and amphetamine These have been amply proved not to be deaminated by aminase under conditions similar to these experiments (2, 3 4)

85

The Warburg apparatus was used for the manometric measurement of oxygen uptake during the deamination. The temperature of the bath was 38.4°C. The flasks were shaken at a rate of 100 times per minute and at a stroke of 4 cm. The flasks contained:

1.7 cc. liver homogenate (aminase)
0.1 cc. M/25 NaCN (fresh)
0.2 cc. M/4 amine (or water for controls)
—————
2.0 cc. total volume

The aminase was actually a homogenate of guinea pig liver since no attempt was made to isolate the enzyme from the tissue except in a few specific instances.[4] The liver of a healthy young guinea pig was removed immediately after killing the animal by a sharp blow on its neck. The liver was washed in cold water to remove blood, the heavy connective tissue of the bile ducts cut away and the organ weighed. The liver was cut into small pieces and homogenized thoroughly for 20 minutes with a little powdered glass and an equal weight-volume of distilled water, using the Potter and Elvehjem technic (5) for the homogenization. The long period of homogenization was to permit the cells to rupture or their permeability to be altered by the distilled water. Another weight-volume of M/8 sodium-potassium phosphate buffer, pH 7.2, was then stirred into the homogenate and the suspension strained through muslin. This gave a very active liver suspension which did not tend to settle out rapidly. For the experiments such as were portrayed in the illustrations *this liver homogenate was always used immediately after it was prepared.* For us this factor *of freshness of the preparation was of the greatest importance for the obtaining of uniformly reproducible and interpretable results.* Before we recognized this fact practically all our earlier experiments were performed using homogenates that had been allowed to remain in the refrigerator at 0°C. for 12 to 18 hours before use. This latter procedure gave liver preparations having even more vigorous oxygen uptakes in the presence of many of those amines found to be oxidized, notably in the case of phenylethylamine added as a substrate.

However, the character of the curves representing the rates of reaction and the oxygen uptakes per mol. of substrate indicated that much more than simple deamination was taking place. We were not certain of the reactions we were measuring. Consequently we have discarded from this report all early experiments and have presented only the work done using the aminase preparations as first described above. We believe that this same physiological factor is primarily responsible for certain qualitative differences between our results and those recently reported by Alles and Heegaard (6) who described and used aminase preparations which were almost necessarily over 24 hours old. We have not found it necessary to place as great emphasis on unrelated fluctuations of the rate of oxidation of various amino compounds with alterations of pH of the medium or other less determinable factors.

Six to ten or more determinations were made of the rate of deamination of each of the amines listed. The compounds were arranged into groups of four representing related alterations in the molecular and compared with tyamine which was always used as a reference compound. The experiments were run in duplicate for each amine at a given time. Fresh sodium cyanide was used to fix the aldehyde, abolish the cyanide-sensitive respiration of the tissue, prevent autoxidation of the compounds, and rule out any effect the compounds might have on the cyanide-sensitive metabolic systems in the cells. Proof that the pur-

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[4] To make sure that an adequate concentration of enzyme was outside the cell a few preparations were centrifuged to remove the cells from the suspension. The supernatant liquor catalyzed the oxidation of phenylethylamine and tyramine at the same rates as the whole liver suspension, demonstrating that availability of the enzyme due to differences of cell permeability to the amines was not a limiting factor in the rates of deamination in these studies.

poses of the NaCN were adequately served was the almost stochiometric uptake of one atom of O_2 per mol of substrate where the deamination was permitted to go to an end. Filter paper saturated with 10% KOH was contained in the center cups of the flasks. Data represented in all the figures have been corrected for the residual O_2 uptake of the duplicate control liver homogenates. In these controls there was substituted for the 0.2 cc. solution of the substrate a corresponding volume of water. Duplicate control determinations of O_2 uptake of the homogenates were run each time a Warburg experiment was set up, even though only 2½ to 3 hours may have elapsed from the beginning of the first of two sets of determinations to the beginning of the second in which the same homogenate was refrigerated at 0°C between experiments.

EXPERIMENTAL RESULTS. Under the conditions outlined above we have obtained easily reproducible results having a low percentage variation from experiment to experiment. This method yields oxygen uptakes of one atom (approximately 139 c.mm.) per molecule of compound deaminated, where deamination took place and the reaction was permitted to go to an end. The equation for this reaction was
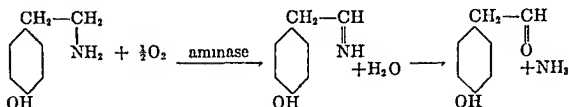
From a first glance at the figures representing the rates of oxidation of these compounds it would appear that almost any substitution into the phenylethyl amine structure altered the rate of deamination in an unpredictable manner. However, careful study of these curves will reveal a very systematic relationship of structure to rate of reaction. The compounds have been grouped according to the way they fit into certain general patterns of the structure rate relationship.

β Phenylethylmethylamines were more rapidly deaminated than β phenylethylamines. To substantiate this generalization compare the curves in figure 1 of β phenylethylmethylamine (3) with β phenylethylamine (6). The secondary amino compound was completely deaminated in about 90 minutes whereas the primary amino compound was only about 26% oxidized in 2 hours. On comparing β methylphenylethylmethylamine (2) with β methylphenylethylamine (6) it may be seen that much the same relation holds as for the above compounds. Comparing β,β dimethylphenylethylmethylamine (7) with β,β dimethylphenylethylamine (8) it is apparent that the rate of reaction was very slow for the secondary amine but that the corresponding primary amino compound was not deaminated over a period of 2 hours to any measurable extent.

Compounds having an unsubstituted phenyl nucleus beta to the amino group had a slower rate of deamination than the corresponding amines having a para phenolic nucleus. Rate of deamination of β phenylethylamines was further increased by the addition of an aliphatic hydroxyl group or inhibited by the addition of two methyl groups to the beta carbon atom. Compare all the curves in fig. 1 to that for tyramine (1) and to the curves for the compounds illustrated in figure 2. In every instance, where there was no aliphatic hydroxyl group the compounds hav-
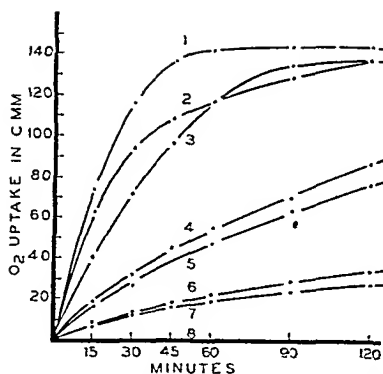
FIG. 1. A COMPARISON OF THE RATES OF OXYGEN UPTAKE IN THE DEAMINATION OF NON-PHENOLIC SYMPATHOMIMETIC AMINES WITH THAT OF TYRAMINE IN THE PRESENCE OF LIVER AMINASE

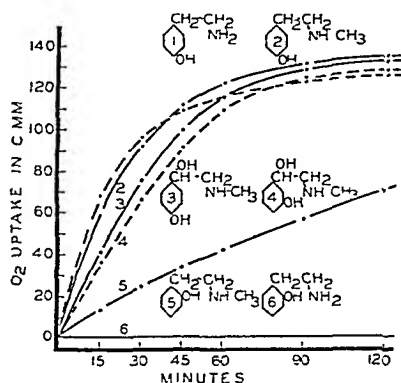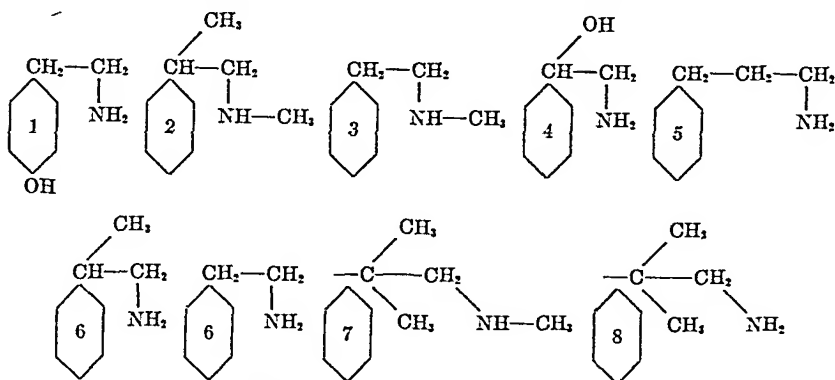The numbers on the curves correspond to the following compounds:

FIG. 2. THE RELATION OF THE POSITION OF THE PHENOLIC HYDROXYL GROUP TO THE RATE OF ENZYMIC OXIDATIVE DEAMINATION OF SYMPATHOMIMETIC AMINES

In all these figures approximately 139 c.mm. of oxygen is equivalent to 1 atom of oxygen per molecule of substrate

88

ing a *para* phenolic nucleus were more rapidly deaminated than those having no hydroxyl group on the benzene ring   This was not because of an oxidation of the phenolic hydroxyl group, for the total O_2 uptake did not differ significantly from that of other phenyl derivatives whose deamination went to completion in two hours   Nor was the difference due to availability of the enzyme to the substrate because of differences in cell permeability to these compounds   Also the known phenolases are cyanide sensitive

Comparing the curves for γ phenylpropylamine (*o*) with β phenylethylamine (6), figure 1, the effect of the proximity of the benzene ring to the amino group on the rate of deamination is evident   However, it would be unwarranted to con clude that the rate of deamination increases progressively as the distance between the aromatic nucleus and the amino group increases   Ethylamine and propyl amine are not deaminated by aminase (2, 6)

β Phenylethanolamine was consistently deaminated more rapidly than β phenylethylamine—see figure 1   When two methyl groups were substituted on the carbon atom *beta* to the amino group the rate of deamination was markedly reduced   To substantiate this the rates of reaction for β phenylethylamine (6) may be compared with β,β dimethylphenylethylamine (8) and β phenylethyl methylamine (3) with β,β dimethylphenylethylmethylamine (7) in figure 1

As the position of the single aromatic hydroxyl radical approached the side chain from the p to o position the rate of deamination of the aliphatic amino group dim inished   These comparisons are made in figure 2   Considering first the com pounds having a *para* phenolic nucleus, the rates of oxygen uptake by the primary and secondary amino compounds (curves 1 and 2) were essentially the same   This is to be contrasted with similar observations for the homologous compounds having no hydroxyl group on the nucleus   In the case of these compounds the aliphatic hydroxyl group significantly decreased the rate of deamination of the side chain   (Compare curve 2 with curve 3, figure 2)

Unfortunately we have no absolute comparisons of the rate of deamination of *meta* hydroxyphenyl with *para* hydroxyphenyl compounds   The comparison of the rate of deamination of synephrin (3) with neosynephrin (4) is complicated by the presence of the aliphatic hydroxyl group on the same C atom as the phenyl nucleus   We would expect this aliphatic hydroxyl group to influence materially the difference between the rates of deamination of the para and meta phenolic derivatives   However, the influence of the change in position of the aromatic hydroxyl group on deamination persisted in spite of the character of the side chain, for the rate of oxidation of synephrin (3) was invariably greater than for neosynephrin (4)   The difference was slight, as was to be expected   The important point is that the difference actually existed

The more clearly demonstrable differences in rate of reaction of *para* and *ortho* phenolic homologs is consistent with the decrease in rate of deamination of a *meta* phenolic compound as compared with its *para* phenolic homolog   One has only to compare curve (1) with curve (6) and (2) with (5), figure 2, to note this marked reduction in rate of deamination of the ortho hydroxyphenylethylamino compounds   Here again, as the influence of the phenolic group decreased the

difference in rate of reaction of the primary and secondary amino compounds became apparent. (Compare curve 5 with curve 6, figure 2).

The catalytic effect of aminase on deamination of tertiary amino compounds was studied but not recorded in the figures. The compounds used were *1*) β-methylphenylethyldimethylamine, *2*) β,β-dimethylphenylethyldimethylamine and *3*) β(4-hydroxyphenyl)-ethyldimethylamine (hordenine). None of these compounds was deaminated in the presence of aminase at a rate measurable by our methods. Looking back at the equation for the deamination of these compounds it is apparent that it would be difficult for tertiary amines to be converted into the corresponding imino compound during the reaction. The methyl groups would be unlikely to combine with oxygen under these conditions as hydrogen ions are known to do.

Sympathomimetic amines having a catechol nucleus and a β-phenylethylamine side chain were rapidly deaminated in the presence of aminase. Comparing the rates of reaction of compounds in figure 1 with the curves for similar amines in figure 2
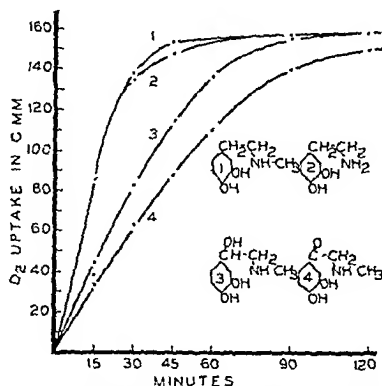
Fig. 3. The Relation of Substitutions into the Side Chain on the Rate of Oxygen Uptake in the Deamination of 3,4-Dihydroxyphenolic Sympathometic Amines

and these in turn with the compounds represented in figure 3 one outstanding generalization seems permissible. In general, β(4-hydroxyphenyl)-ethylamines were deaminated more rapidly than β-phenylethylamines and β(3,4-dihydroxyphenyl)-ethylamines were more rapidly deaminated than the corresponding mono phenolic compounds.

Just as was noted for the previous groups of compounds, the aliphatic hydroxyl group diminished the rate of deamination of adrenalin (*3*) as compared with epinine (*1*) which does not have this radical in its molecule. Consistent with this observation, the ketone of adrenalin (*4*) was more slowly deaminated than was adrenalin (*6*) itself. As in the case of the *para* phenolic homologs, there was no appreciable difference in the rate of deamination of the primary and secondary amino compounds having no aliphatic hydroxyl or ketone substitutions, curves 1 and 2, figures 2 and 3. The slightly greater than theoretical oxygen uptake for these catechol compounds is probably due to some autoxidation in spite of the presence of cyanide.

INTERPRETATIONS Such a systematic relationship between molecular struc
ture and rate of enzymic deamination of sympathomimetic amines has not been
recognized heretofore   Though our observations are of an orderly arrangement of
reaction rates according to structure, the interpretation of intramolecular forces
which determine our results is quite difficult and is not yet complete   To explain
our findings we have turned to the physical aspects of organic reactions and our
limitation has been the inadequacy of information in the field which we have
found applicable to this problem   We are primarily concerned here with the
rate of oxidation of primary or secondary amino groups

In considering the difference in rate of oxidation of primary and secondary
amino compounds we must recognize that here we deal with two different but
similar types of substrate   It is infrequently recalled that a secondary amine
may correctly (7) be considered to partake of the nature of an imino group,
=NH   Referring back to the equation for the deamination of primary amines
it will be seen that the first step in their deamination is the oxidation to the
corresponding imino compound   This type of imino group, where both the
covalent bonds are shared by a single carbon atom, is quite unstable and undergoes spontaneous hydrolysis in the presence of water with the splitting off of am
monia   In the case of secondary amines where two covalent bonds of the imino
group are shared by different carbon atoms this bonding influence is greater than
where they are shared by a single carbon atom   This tends to make for a
somewhat greater stability of the molecule in the latter instance   However, as
the curves in figure 1 illustrate, the imino nature of the secondary amines was
consistently evident for these compounds were more easily oxidized (rate of reaction was faster) than were the primary amino compounds

Because of their relative lability, the reactivity of the secondary amino com
pounds was influenced more easily and to a greater extent than the primary
amines by changes in the side chain, figure 1   It should be noted that the
initial rate of deamination of β methylphenylethylmethylamine was greater than
for β phenylethylmethylamine whereas the rate of reaction of the corresponding
primary amines was so nearly the same as to be reproducible by a single curve,
figure 1   The addition of a methyl radical to the beta C atom conveys to it the
properties of an asymmetric C atom   It is the asymmetry of this C atom which
confers on the imino group its greater lability, yet this effect is so slight as not
appreciably to influence the reactivity of the amino group of the β methyl phenyl
ethylamine

When a second methyl group was added to the beta C atom as in β dimethyl
phenylethylamine and β,β dimethylphenylethylmethylamine, it conferred on
that C atom the greatest symmetry, and hence stability, of the molecule possible
without replacing the phenyl ring by CH_3 or by substituting 3 phenyl rings about
this C atom   Because of this increased stability these two compounds reacted to
deamination more nearly as would ethylamine and ethylmethylamine respec
tively   It is known that ethylamine is not deaminated in the presence of amin
ase (2, 6)   The imino characteristic of the secondary amine persisted in this
case but even so its reactivity was reduced to a rate of 27 c mm oxygen uptake
in two hours

The aliphatic hydroxyl group of β-phenyl-β-hydroxyethylamine produces an asymmetry of atoms or groups about the C-atom *beta* to the amino group. The effect of this asymmetric arrangement on the reactivity of the amino group is complicated by the fact that the hydroxyl group carries with it a dipole moment of approximately equal magnitude and opposite sign to that of the primary amino group. Actually, at least three factors together determine the rate of deamination of this compound: *1*) the proximity of polar groups of opposite sign, *2*) the magnitude of the asymmetry about the beta C-atom, and *3*) the presence of equal quantities of optical isomers in the synthetic compound used for these studies. The first factor would in itself tend to slow the rate of deamination since the resultant moment decreases to approach zero when polar groups of opposite sign approach each other. However, the position of the aliphatic hydroxyl group is such as to produce a much greater degree of asymmetry about the *beta* C-atom than was present when the relatively non polar methyl group was present in this position. This greater asymmetry would in itself increase the lability of the amino group. In synthetic compounds of this type the factor of optically active forms tends to cancel itself. The resultant of these complex internal forces was to produce only a moderate increase in reactivity of the β-phenyl-β-hydroxyethylamine.

To explain the greater rate of deamination of γ-phenylpropylamine than of β-phenylethylamine we must consider the effect of the introduction of a benzene ring into the molecule on the reactivity of an aliphatic amino compound. Neither ethylamine nor propylamine is deaminated in the presence of aminase (2, 6). The aromatic nucleus, of itself, has no appreciable dipole moment but when it is substituted into a saturated aliphatic compound it produces a degree of asymmetry which influences the polarity of other groups in the molecule. This effect of the aromatic nucleus is acyclous, activating an electron drift toward it from the amino nitrogen, decreasing the bonding attraction between nitrogen and the proton hydrogen, hence facilitating the transfer of 2 hydrogen atoms to oxygen in the presence of aminase. The magnitude of this intramolecular effect is increased somewhat when oppositely polar groups are separated each from the other's immediate field of attraction, as by one or two carbon atoms. This latter fact is borne out by, or may be used to interpret out observations of, the slower rate of oxidation of β-phenylethylamine than γ-phenylpropylamine.

The markedly greater rate of deamination of the p-hydroxyphenylethylamines over their counterparts having no aromatic hydroxyl group may be visualized fairly simply as follows. The aromatic hydroxyl group possesses an inherent dipole moment opposite in sign and of similar magnitude to that of the amino group. The phenolic ring as a whole may be considered as assuming the polarity of the hydroxyl group. But, where the phenolic nucleus is attached to a side chain the magnitude of the moment exerted by the hydroxyl group, hence the whole nucleus, varies with its position with respect to the oppositely polar side chain. Consequently the molecular moment of the compound is determined by the vector addition of the polarities at the two points of substitution on the ring.

The formula (8) for this is

$$\mu = \sqrt{\mu_1^2 + \mu_2^2 + 2\mu_1\mu_2 \cdot \cos\theta}$$

where μ is the molecular moment in this case, μ_1, the moment of positive sign due to the side chain, μ_2 is the negative moment of the hydroxyl group and θ is the angle separating the two poles as determined by the ortho, meta or para position of the hydroxyl group. By assigning an arbitrary value of $+ 1 \times 10^{-18}$ to the side chain (μ_1) and the determined value of 1.83×10^{-18} to the OH group (μ_2) it can be shown that the moment μ decreases as the hydroxyl group approaches the side chain from the para to the ortho position

The negative polar influence of the phenolic ring, then, is of greatest magnitude for the *para* compounds and least for the *ortho* homologs. This negative pole is
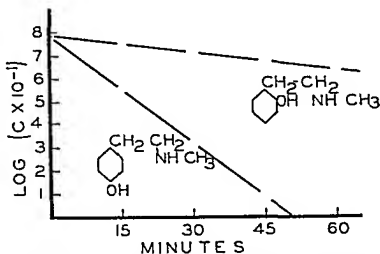
Fig 4 Demonstrating that the Enzymic Deamination of Structurally Similar Monophenolic Sympathommetic Amines Was a First Order Reaction, and That *the Difference in Position of the Hydroxyl Group Markedly Altered the Rate* but not the Character of the Reaction

Data for the coordinates of these curves were taken from the corresponding curves in figure 2 The initial concentration of substrate was 6.25×10^{-2} mol

electron attracting and acyclous, which causes an electron drift to it from the amino nitrogen This effect is responsible for the decreased electronic stability of the amino group, and the magnitude of this negative force or "electron sink" (9) determines the rate of oxidation of that group [5] When the hydroxyl group exists in the *para* position as in tyramine or p hydroxyphenylethylmethylamine this electron sink is of such magnitude as to minimize the difference in rate of oxidation of the primary and secondary amino radicals, as seen in figure 2

It is unfortunate that we do not have the *meta* hydroxy homologs uncomplicated by an aliphatic hydroxyl group Figure 4 illustrates that for the phenolic compounds having no aliphatic hydroxyl group the rate of oxidation is that of a

[5] Another possible partial explanation for the low rate of reactivity of the o hydroxy compounds is that of hydrogen bonding between the aromatic OH and the amino group This is a frequent occurrence in homologous orthohydroxy compounds

first order reaction since the log of the concentration of substrate is a perfectly linear function of time. This figure also serves to dramatize the influence of position of the aromatic hydroxyl group on the magnitude of the electronegative polarity of the nucleus and the part it plays in determining the rate of oxidation of the amines. It should be noted in this connection, figure 2, that as the electronegative influence of the ring decreased the difference in reactivity of the primary and secondary amino compounds again became apparent. β-(o-hydroxyphenyl)-ethylmethylamine was oxidized, β(o-hydroxyphenyl)-ethylamine was not deaminated at a measurable rate.

What has been written for monohydroxy phenolic compounds applies also and to even greater extent for their homologs having an *ortho* dihydric nucleus. A comparison of figures 2 and 3 substantiates our interpretation that the greater the magnitude of the electron sink represented by the negatively polar aromatic nucleus the greater the electron drift toward it from the amino nitrogen, the less the attraction between nitrogen and hydrogen and the greater the rate of oxidation of the amino group.

We come finally to the compounds whose rates of reaction are most difficultly interpreted but easily anticipated from the previous discussion—phenolic and catechol homologs having an aliphatic hydroxyl or ketone radical on the carbon atom *beta* to the amino group. A really adequate interpretation of this inhibitory effect seems limited by the complexity of the problem and our understanding of the interplay of the intramolecular influences which determine rate of deamination of these compounds. Such an interpretation should consider *1*) the close proximity of the polar OH to the NH_2 or NH-CH_3 group of similar magnitude and opposite sign, *2*) the effect of the stereoasymmetry introduced by the aliphatic hydroxyl group, *3*) a possible impedance by the aliphatic OH group of the electron drift from the amino group to the negatively polar phenolic or catecholic ring, *4*) the effect of the aliphatic hydroxyl group on the negativity of the electron sink represented by the aromatic nucleus, and *5*) the effect of this electronegative nucleus on the asymmetry of the side chain. Some of these factors have been discussed by us in this and a previous publication (1).

Consistent with the observation that an aliphatic hydroxyl group decreased the reactivity of phenolic pressor amines is another, that adrenalone, which has a ketone group beta to the secondary amino group, was deaminated at a slower rate than adrenaline (figure 3). This was predictable, for an aliphatic hydroxyl group has a dipole moment of 1.83×10^{-18} e.s.u. whereas the moment of the corresponding ketone is 2.79×10^{-18} e.s.u. The sign of the moment is the same in both instances, hence the ketone should and did inhibit deamination to a greater extent than the hydroxyl group.

SUMMARY

From our observations we have offered the following generalizations for the relation of molecular structure to the rate of oxidative deamination of sympathomimetic amines in the presence of aminase.

1. Where a difference in rate was measurable, secondary amino compounds

were oxidized more rapidly than the corresponding primary amino homolog. This difference in rate was minimized when there existed in the molecule a *para* phenolic ring or a *3,4*-dihydroxy catechol nucleus.

2. Where there existed an asymmetrical *beta* carbon atom in the side chain, due either to a single methyl or hydroxyl group, the rate of deamination of the primary or secondary amino group was increased. This generalization together with the next one was limited to compounds having no hydroxyl groups on the aromatic nucleus.

3. Where two methyl groups existed on the *beta* carbon atom, reducing its asymmetry, the rate of deamination was markedly reduced.

4. Tertiary amino compounds were not deaminated to any measureable extent.

5. Where a *para* phenolic radical existed in the molecule the rate of oxidative deamination was markedly more rapid than for its homolog having no phenolic group.

6. If the phenolic group was shifted from the *para* to the *ortho* position the rate of deamination was markedly reduced whereas the order of the reaction remained the same.

7. There was an increase in rate of deamination as one passed from the non-phenolic through the *para* phenolic to the *3,4*-dihydroxy phenolic compounds having an identical side chain.

8. Where an aliphatic hydroxyl or ketone group was introduced on the carbon atom *beta* to the amino group of the phenolic pressor amines the rate of deamination was reduced. This reduction in rate was greatest for the ketone of adrenalin, when one compared the rate of oxygen uptake for epinine, adrenalin and adrenalone (adrenalin ketone).

Wherever possible we have interpreted these reaction rates in terms of variations of intramolecular electronic forces set up by substitutions into the fundamental phenylethylamine structure.

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BIOASSAY OF DIURETICS

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Although many studies have been made of the mechanism of diuresis, there has been no systematic quantitative study done on diuretics. Such an investigation should include a determination of the dose-action relationship of various diuretics by a reliable method, and an estimation of their relative potency with reference to a suitable standard. To be of practical use in future studies, the method should be simple, accurate and applicable to a variety of substances. A method using rats for the estimation of anti-diuretic potency has been described by Burn (1). This method, or a slight modification of it, has been used for diuretic assays by Marx (2), Herre (3), Jaretzky and Neuwald (4), and Vollmer (5). Controversial results reported in the last two citations clearly demonstrate the inadequacy for this purpose of the anti-diuretic assay method, which is devised for the measurement of a decrease in urinary excretion and requires a normally high excretion of urine. For the measurement of an increase, it is desirable to have the normal urinary excretion at a minimum.

In this paper a method suitable for diuretic assays will be described, and some results of its application to the study of several diuretics, most of which are in common use, will be presented.

METHOD The procedure to be described for the determination of diuretic potency of drugs differs from Burn's in four important points: (1) the amount of liquid fed is reduced to half of that used in the antidiuretic method (i.e., 25 cc./kgm. instead of 50 cc./kgm.); (2) isotonic sodium chloride solution rather than tap water is used for feeding, (3) the number of rats used for each dose is increased from four to eight, and finally (4) the total volume of excretion during a definite period of time, rather than the time necessary for the rate of excretion to reach a maximum, is measured as the measured variable. As a result of these modifications, basal urinary excretion is reduced to a minimum, and variations among groups are reduced considerably.

Healthy male Sherman rats weighing 140 to 240 grams were fasted for eighteen hours previous to the experiment, and were then given the substance to be tested in proportion to their weight. When administered orally, the substance was dissolved or suspended in isotonic sodium chloride solution at approximately body temperature, and fed to the animals through a stomach tube. After the feeding, the animals were placed in sub-groups of four in special metabolism-cages (Fig 1), and remained there without food or water for the duration of the experiment. Two sub-groups were used for each dose. At the end of a definite period of time, the animals were taken out of the cages and the total volume of urine excreted by each group was measured. For all of the substances investigated, except bismuth potassium tartrate and salyrgan, a period of five hours was found to be adequate, as indicated by a great decrease in the rate of excretion during the fifth hour. Since, in the process of feeding, the animals lose all their urine, it was considered important at the end of the experiment to expel the urine from the bladders by pulling at the base of the tail.

In most experiments five groups of approximately equal weight were used. One group, as a control, was fed normal saline solution. In most cases two groups were fed suitable

doses of urea, and the remaining groups were given varying doses of the substance to be tested. The volume of urine excreted during five hours was in each case expressed as per cent of the liquid administered.

This percentage gives a measure of *urinary excretion* independent of group weight, and may be used in the calculation of *diuretic action*. The ratio $\dfrac{\text{Urinary excretion in test group}}{\text{Urinary excretion in control}}$ could be used as a measure of diuretic action for a given dose. In practice, however, it was found necessary to compare test groups receiving a new preparation with parallel test groups receiving a standard drug. This reduces the variability materially. Since the same control group appears in the estimation of relative potency only as a constant term which would cancel out, it is simpler to use the percentage of urinary excretion for such estimates. The control then serves as a check on experimental conditions as in the assay of Vitamin A, but does not enter into the calculation of the relative potency. *Diuretic activity* is the diuretic potency of a substance referred to that of the standard, urea, the activity of which is taken equal to one. Urea was chosen as the standard diuretic because
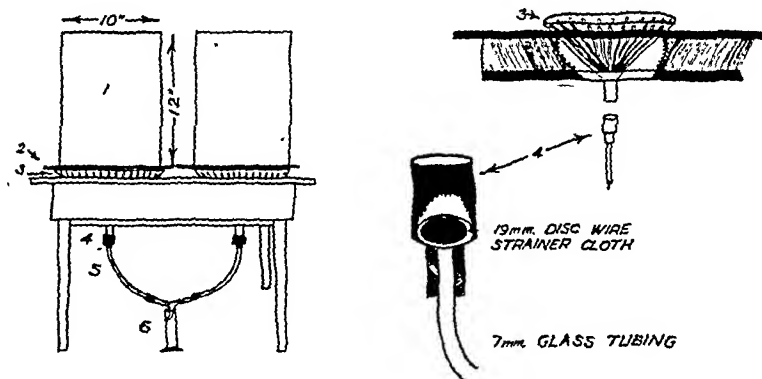
FIG. 1. SKETCH OF THE METABOLISM CAGE

(1) Glass cylinders, 10 inches in diameter and 12 inches high; (2) circular wire screen; (3) rib funnel—1 gallon capacity; (4) vaccine stopper with fine screen inset; (5) glass tubing with "Y" joint; (6) volumetric cylinder.

it is stable *in vivo* as well as *in vitro*, a relatively weak diuretic and nontoxic in very high doses.

Bismuth potassium tartrate was injected intraperitoneally in doses varying from 2.5 to 50 mg./kg., and the proper amount of normal saline was administered orally following the injections. Salyrgan was injected in doses varying from 6 to 30 mg./kg.; and then normal saline or 1% ammonium chloride in saline (4.7 mM/kg.) was fed. Ammonium chloride was chosen for its well known potentiation of salyrgan diuresis (6). When it was used, determinations of diuretic action were made with reference to the urinary excretion of the group receiving ammonium chloride alone. With these two substances it was found necessary to keep the animals on test for approximately twenty-four hours.

In each instance several experiments were done with three or four different doses of the substance to determine the type of dose-action curve to be obtained. Within a fairly large range of doses, most of the substances yielded an almost linear relationship between log dose and log effect. In several instances the curve was approximately linear only within the range of very low doses. The best fitting straight line for each set of data was calculated from the regression equation (I)

$$y = \bar{y} + b\,(x - \bar{x}). \quad\dotfill\quad (I)$$

where $\bar{y}$ is the mean value of y (log effect), $\bar{x}$ the mean value of x (log dose) and b the regression coefficient determined from the equation

$$b = \frac{\Sigma y(x - \bar{x})}{\Sigma(x - \bar{x})^2} \tag{II}$$

In subsequent experiments the effect of two or more doses of the substance was compared with that of one of more doses of urea   The dose action curves of the substance and of urea

TABLE 1

Variability of urinary excretion among groups on same day

Control rats, fed 25 cc /kgm of 0 9% NaCl solution

RATS PER GROUP	EXPER DATE	NUMBER OF GROUPS	URINARY EXCRETION $\frac{\text{VOL EXCRETED IN 5 HOURS}}{\text{VOL ADMINISTERED}} \times 100$									Mean
			Original data									
	1941											
4	9–19	4	34 3	23 8	32 3	32 4						30 7
	9–30	8	21 5	32 6	30 5	40 2	16 8	33 9	19 6	24 2		27 4
	10–8	8	13 8	12 0	14 4	22 4	19 3	7 2	14 3	41 1		18 1
	67 more exper	2										
Average												32 7
8	12–29	2	23 0	29 7								26 4
	12–31	5	30 4	23 3	43 5	40 8	41 8					36 0
	1942											
	1–5	5	37 2	22 3	32 0	35 0	36 1					32 5
	1–7	3	50 5	52 1	47 2							49 9
	1–14	2	33 4	29 6								31 5
	1–15	3	33 1	35 4	28 3							32 3
Average												31 6

Analysis of Variance

RATS PER GROUP	VARIATION	DF	SUM OF SQUARES	MEAN SQUARE OR VARIANCE	F VARIANCE RATIO
4	between days	69	15487 48	224 46	2 595
	within days	84	7265 795	86 5	
8	between days	5	899 83	179 97	4 85
	within days	14	519 56	37 11	

were plotted together   When the dose effect curves are parallel within the experimental error, the distance along the abscissa between the curve for urea and that for the substance gives the logarithm of the diuretic activity of the substance

RESULTS   *Controls*   In the description of the method, special emphasis was placed on the necessity for using a sufficient number of rats in each group, and comparing the urinary excretion of the test groups with that of the control group of the same day, to ascertain that diuresis is obtained   Results presented in Table 1 give the comparison of the urinary excretion for groups on the *same day*

and on *different* days.   The analysis of variance shows that the variation between days is significantly greater than between groups tested on the same day.   As would be expected, the variance within days for groups of 8 rats is about one-half of that for groups of 4 rats.

Since the animals are fed a considerable amount of liquid at one time, it was of great importance to choose a liquid which is normally retained in the body to a large extent throughout the duration of the experiment, and which does not obscure the action of diuretics.   Isotonic sodium chloride was found to fulfill these requirements.   Substitution of Ringer's solution for saline does not significantly alter the value of urinary excretion.   Substitution of tap water, however, more than doubles it (Table 2).

Urea.   Since urea was chosen as the standard, it was more thoroughly studied than any other substance.   The results obtained are summarized in Tables 3–5. Eight curves, each with three or four points, were obtained on different days using 8-rat groups.   The data are given in Table 3.   The variation in the effect

TABLE 2

Effect of substituting Ringer solution or tap water for saline solution on urinary excretion
Groups of eight rats each

EXPERIMENT DATE	URINARY EXCRETION						AVERAGES	
	Ringer solution			Control (saline)			Ringer solution	Control
1/ 7/42	54.1	37.5	42.5	50.5	52.1	47.2		
1/14/42	30.1	25.6		33.4	29.6			
1/15/42	33.1	30.0	50.2	33.1	35.4	28.3	36.8	37.9
	Tap water			Control (saline)			Tap water	Control
9/19/41	59.9		63.2	29.1		32.4		
12/ 9/41	65.4		65.5	23.0		29.7	63.5	28.6

expected for each day at the average dose of urea (25.3 millimoles per kg.) is greater than that in the slope of the dosage-effect curves, when both are compared with their respective errors.   This difference in average susceptibility has been adjusted so as to bring the mean response-mean log-dose for each individual curve on the same line, Fig. 2, with a slope of b = .7005.   This value agrees well with that obtained for 55 curves, each based on only 2 points (b = .7209) (Table 4, second row).   The individual curves are relatively stable in slope with a smaller variance than that of individual readings about 7 of the 8 curves in Table 3.   One curve, that on 11–30–42, was excessively variable.   The mean slope in 63 experiments is 0.710 ± .026.   An experiment was done with four different doses of urea on 4-rat groups, using the Latin-Square arrangement, and the results were analyzed by Yates' method (7) (Table 5).   The variation due to the slope of the curve (b = .718) was greatest, that due to the difference in days was nearly 3 times as large as the error, but differences between rat groups and about the straight line were less than the experimental error.

Sodium, Potassium and Ammonium Salts Of the five salts studied, potassium nitrate, potassium acetate and ammonium chloride are in common use as di-

TABLE 3

Dose action curves from single experiments with 3 or 4 doses of urea, dose in millimoles of urea per kilogram of rat

DATE	Saline control	With log-dose of urea					SLOPE b	STANDARD DEVIATION ABOUT LINE s	LOG-EFFECT AT MEAN LOG DOSE OF 1 4025
		1.097	1 274	1 398	1 574	1 699			
11- 5-41	1 617	1 769	1 867	1 898	2 064		595	0328	1 946
12-16-41	1 698	1 812		1 995	2 163	2 222	703	0236	2 021
12-17-41	1 643	1 855		2 037	2 152	2 288	697	0282	2 056
3-30-42	1 428	1 762*	1 815†	1 912			848	0232	1 893
11-27-42	1 732	1 655		1 785	1 949	2 179	821	0583	1 865
11-30-42	1 405	1 767		1 731	1 996	2 155	643	1328	1 885
12- 1-42	1 555	1 718		1 830	2 036	2 191	776	0724	1 916
12- 2-42	1 396	1 805		1 957	2 054	2 192	615	0401	1 975
Combined value							7005	0683	1 9446

* Log dose = 1 223

† Log dose = 1 318
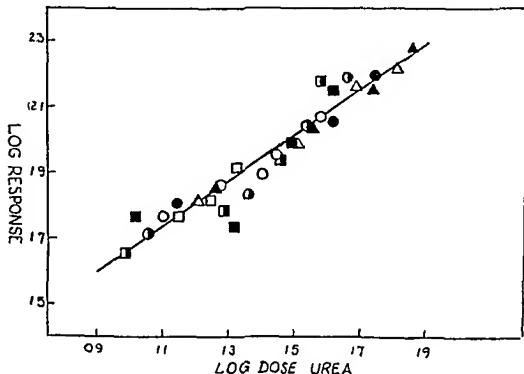
FIG 2 DOSAGE RESPONSE CURVE FOR DATA ON UREA IN TABLE 3

The experiments on different days have been adjusted to an equal susceptibility for plotting by adding a constant term to the log doses for each day Each day s results are shown by a different symbol

uretics, but not sodium nitrate or sodium acetate The original data for potassium and sodium nitrate are given in Table 6, with the log ratio of potencies (M)

TABLE 4

Analysis of variance between slopes of dose-action curves for urea in Table 3 and of 55 curves with 2 doses, not shown in detail; eight-rat groups on each dose

VARIATION DUE TO	DEGREES OF FREEDOM	MEAN SQUARES
Combined slope, all 63 curves of $b_c = .71048$	1	1.34948
Difference between 2-dose and multiple-dose slopes..	1	.00028
Difference in slopes of individual curves .	61	.00168
Scatter of observations about 7 of 8 separate curves in Table 3..... .	13	.00267
Scatter of observations about curve for 11–30–42 .	2	.01763

TABLE 5

Experiment with 4-rat groups arranged in a Latin square as a test of sources of variation; highly variable controls omitted in analysis of variance by Yates' method

DATE	DIURETIC RESPONSE IN LOG OF % EXCRETION					TOTAL	SLOPE b
	d	a	o	b	c		
12/8	2.075	1.790	(1.612)	1.902	1.989	7.756	.604
	c	o	b	d	a		
12/14	2.093	(1.899)	2.037	2.135	1.821	8.086	.645
	o	c	d	a	b		
12/18	(1.882)	1.853	2.150	1.597	1.982	7.582	1.019
	a	b	c	o	d		
12/23	1.954	2.088	2.022	(1.814)	2.157	8.221	.364
	b	d	a	c	o		
12/28	1.738	2.094	1.655	1.930	(1.213)	7.417	.960
Total.. .	7.860	7.825	7.864	7.564	7.949	39.062	Mean. .718

Dose of urea

	SYMBOL				
	o	a	b	c	d
	Log mM				
		1 097	1 273	1 398	1 574
Diuretic response in log of % excretion					
Total	8.360	8.817	9.747	9.887	10.611
Mean .	1.6720	1.7634	1.9494	1.9774	2.1222

Analysis of variance

VARIATION DUE TO	DEGREES OF FREEDOM	MEAN SQUARE	F
Difference between groups of rats	4	.00534	.60
Difference between days of test	4	.02512	2.82
Slope of regression line	1	31361	35 24
Curvature about line	2	.00616	.69
Error. . .	8	.00890	1.00

computed for each assay The data were first analyzed for the parallelism of their dose response curves with the results shown in Table 6a The small variance with few degrees of freedom about the individual curves is apparently not characteristic of the variation between curves Although the 5 groups in Table 6 differ more in slope than the curves comprising the groups, the difference is not significant Therefore, all observations have been combined to obtain a standard

TABLE 6

Dose action curves of potassium nitrate and sodium nitrate and their activities relative to urea
M is log ratio of potency relative to urea, M¹ that relative to KNO_3

GROUP	DRUG	LOG	LOG EXCRETION ON				GROUP	DRUG	LOG	LOG EXCRETION ON		
			10-1	10 2	11 10	11 11-41				12 1	12 2	12 3-41
		mM/kg							*mM/kg*			
1	KNO_3	297			1 682		2	$NaNO_3$	301	1 610		
		393		1 731		1 770			469		1 677	1 735
		598			1 798				582			1 820
		694		1 989		1 945			602	1 794		
		773			1 961				770	1 933	2 030	2 000
		787	2 013						946		2 196	2 163
		870				2 061						
		1 092	2 266									
	Urea	1 319	1 863	1 828	1 863	1 845		Urea	1 319	1 904	1 901	
	Control		1 580	1 561	1 625	1 633		Control		1 486	1 584	1 539
	M		722	815	702	766		M		606	670	
			12 9	12 10	12 11-41					12-9	12 10	12 11 41
3	KNO_3	393	1 618	1 493	1 808			$NaNO_3$	389	1 595	1 553	1 598
		870	2 066	2 048	2 106				866	1 899	2 035	2 106
	Control		1 428	1 386	1 644			M¹		– 114	033	– 126
			11 20	11 25	12-3-42					12-9	12 11	12 17-42
4	KNO_3	490		1 705	1 742		5	$NaNO_3$	469	1 733		
		570	1 709						645		1 724	1 761
		787		2 066	1 938				770	1 871		
		870	1 936						946		1 957	2 022
	Urea	1 097	1 636	1 791	1 810			Urea	1 097	1 843	1 734	1 815
		1 398	1 886	2 012	1 903				1 398	2 028	1 931	1 987
	Control		1 303	1 687	1 742			Control		1 695	1 632	1 694
	M		604	589	588			M		462	462	440

deviation of s = 0616, a combined slope of b = 0 8053 and λ = 0765 The log ratio of potencies and its error is computed for each day with the combined slope In this way the results are more consistent than when computed from the slopes for each group of tests (Table 7) The potencies of both KNO_3 and $NaNO_3$ relative to urea shift significantly between groups 1–2 and 4–5 in Table 6, but retain the same relation to each other throughout all tests as shown in the right-hand column of Table 7

The dose-response curve of potassium acetate has the same slope as that of urea, so that its relative potency (avg. 3.35) is independent of the level of urinary excretion at which it is measured (Table 8). The dose-response curves of sodium acetate are, however, significantly steeper ($b = 1.1194$) than the corresponding curves of urea and potassium acetate (average $b = 0.6085$). In this case, there-

TABLE 6a

Analysis of variance for parallelism of dose-response curves in Table 6

VARIATION	DEGREES OF FREEDOM	VARIANCE
Between slopes of dif. groups..........	4	.00599⎫
Between slopes within groups on dif. days................................	11	.00352⎬ $s^2 = .003794$
Between slopes of dif. drugs on same day..................	9	.00316⎭
About 3 and 4 dose curves.............	6	.00056

TABLE 7

Mean log-ratio of potencies based on Table 6

UREA AS STANDARD				POTASSIUM NITRATE AS STANDARD			
Group	Diuretic	$M \pm s_m$	Relative potency	Groups	Diuretic	$M \pm s_m$	Relative potency
1	KNO₃	.751 ± .046	5.63	1–2	NaNO₃	−.113 ± .078	.77
4	KNO₃	.594 ± .044	3.92	3	NaNO₃	−.069 ± .044	.85
2	NaNO₃	.638 ± .063	4.35	4–5	NaNO₃	−.139 ± .063	.73
5	NaNO₃	.455 ± .044	2.85				

TABLE 8

Dose-action curves of potassium acetate and its activity relative to urea

DRUG	LOG	LOG % EXCRETION ON						MEAN ACTIVITY
		10–3	11–7–41	12–21	12–22	12–29	12–30–42	
	mM/kg.							
K acetate	.582	1.796	1.723	1.774	1.927	1.609	1.772	
	.805	1.951						
	.883		1.899	1.923	2.038	1.916	2.002	3.35
	1.059		1.981					
Urea	1.097			1.668	1.888	1.784	1.843	
	1.319	1.940	1.817					1.00
	1.398			2.005	1.945	1.945	1.985	

fore, it is necessary to compute the relative potency from the data in Tables 9 and 10 at a specified level of log % excretion which is taken at 1.895. At higher levels of excretion, the relative potency of Na acetate would be greater relative to urea than at lower levels. In the later experiments it was significantly less than in the first tests in 1941.

The dose-response curve of ammonium chloride has the same slope (b = .8085, s = .0642) as that for urea (b = .6191, s = .1416), so that its relative potency, 2.7, is independent of the level of urinary excretion at which it is measured (Table 11).

Xanthine Diuretics. With theophylline and theobromine, an approximately linear relationship between log dose and log action is obtained only in the lower range of doses, "overdosage" producing a decrease in diuretic action. With caffeine, the measurable dose range is still more restricted. Calculation of the

TABLE 9

Dose-action curves of sodium acetate and its activity relative to urea and to potassium acetate

DRUG	LOG	10-6	11-6	11-26	11-27	11-28	12-4	12-5	12-8	12-12-41	1-6	1-7-43
				1st group					2nd group			3d group
Na ace-tate	mM/kg .662										1 557	
	.711						1.728	1.584	1.671	1.809		
	.741		1.611			1.766						1.530
	.866			1.915	1.930	1.940						
	.964	1.865			2.088	2 062					1.944	
	1.042		1.951	2.084	2 152							1 871
	1.063						2.017	2 032	2.000	2.161		
	1.167			2 253								
	1.220		2.118									
	1.265	2.152										
K ace-tate	.707						1 972	1.856	1.900	1.856		
	1 059						2.145	2.088	2.024	2 141		
Urea	1 097										1.856	1.671
	1 319	1 807	1 888	1.934	1.870	1.948						
	1.398										1.975	1.918

Mean activity of Na acetate
    relative to urea   (1st group)   2.762 ± .260
    relative to K acetate   (2nd group)   .785 ± .045
    relative to urea   (3d group)   .   1.854 ± .106

diuretic activities is, therefore, possible only approximately. They are: theobromine, 7.2; caffeine, 32; and theophylline, 115. Theobromine in higher doses was fed in suspension, and in such doses it proved after 24 hours to be definitely toxic. With theophylline and caffeine, the doses could be increased to the desired extent, and the effect of overdosage could be studied in entirety. The highest dose used of theophylline produced a decrease in urinary excretion below that of the controls Even with the lowest doses of theophylline and caffeine, there were definite manifestations of central excitation, but no such signs could be noticed with theobromine.

Bismuth Potassium Tartrate. The preparation used, with a Bi content of 62%, has a diuretic activity of approximately 219. High doses produce a decrease in urinary excretion, but even with diuretically active doses, albuminuria and glycosuria are observed.

Salyrgan. Salyrgan was tested alone and in combination with ammonium chloride. The results, given in figure 3, show salyrgan to be a potent diuretic

TABLE 10

Dose-action curves of sodium acetate and its activity relative to urea on 4-rat groups in the Latin Square arrangement

Since the analysis showed the differences between days relatively unimportant the results were handled as a randomized block.

GROUP	Saline control	LOG % EXCRETION			
		Acetate		Urea	
		808	1 109	1 097	1.398
2	1.683	1.495	2.062	1.601	1.743
3	1.515	1.718	2.066	1.651	1.894
4	1.511	1.745	1.969	1.830	2.063
5	1.511	1.750	2.099	1.940	1.931
6	1.367	1.715	2.119	1.964	1.818
7	1 185	1.639	1.949	1.704	1.931
9	1.750	1.643	2.146	1.828	2.017
10	1.448	1.772	2.109	1.693	1.905
Total. .	11.970	13.477	16.519	14.211	15.302
Mean	1.4963	1.6846	2 0649	1.7764	1.9128

Relative potency = 1.982 ± .101.

Analysis of variance

	D F	MEAN SQUARE	F
Groups.. .	7	.020980	2.76
Controls vs. treated	1	.845210	111 27
Acetate vs. urea	1	.007290	.96
Combined slope	1	533803	70.27
Parallelism ..	1	.118950	15.66
Groups × control vs. treated	7	.028670	3.77
Error .	21	.007596	1.00

within a very narrow range of doses. A slight overdose produces a definite decrease in urinary excretion. The active range is seemingly narrower without ammonium chloride and the optimum dose slightly higher. When ammonium chloride was used with salyrgan, in several instances the diuretic effect could be observed after five hours, and in nearly all instances, after ten hours. Without ammonium chloride, it took somewhat longer for the diuretic effect of salyrgan to become manifest. Within the range of diuretic doses, albuminuria was observed

in all instances and glycosuria in samples collected after ten hours   In the lower dose range, linear relationship was again obtained between the logarithm of the

TABLE 11

Dose action curves of ammonium chloride and its activity relative to urea

DRUG	LOG	LOG % EXCRETION ON								MEAN ACTIVITY
		2 23	3 2	3-20	3-23	3-24-42	1 13	1 14	1 18-43	
NH₄Cl	*mM/kg*									
	670	1 646								
	711									
	846			1 096	1 461	1 557	1 850	1 833	1 647	2 7
	971	1 932	1 826	1 718						
	1 012						2 099	2 065	1 936	
	1 147		2 040	1 959	1 720	1 625				
Urea	1 097						1 731	1 735	1 724	
	1 319	1 838								1 0
	1 398						1 987	2 929	1 833	
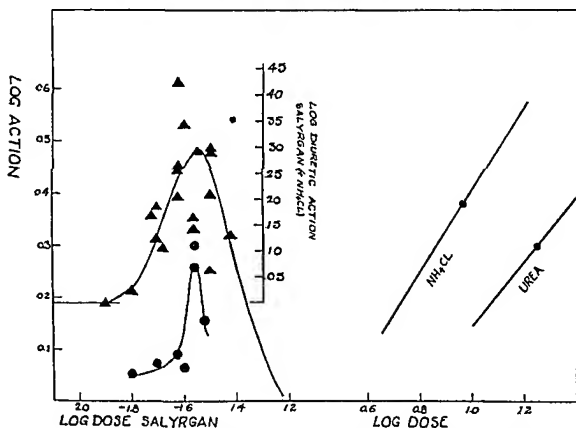
FIG 3 DOSE ACTION CURVES OF AMMONIUM CHLORIDE SALYRGAN AND SALYRGAN WITH 4 67 mM/KG AMMONIUM CHLORIDE OVER A WIDE DOSE RANGE
● salyrgan   ▲ salyrgan with NH₄Cl

dose and the logarithm of action   The diuretic activity was found to be about 400

Biuret. A number of substances which were considered likely to show some diuretic activity were studied. One of these is biuret which has a formula closely related to that of urea and is similar to it in some respects.[1] The original data obtained with low doses are given in Table 12. With higher doses necessitating a concentration greater than 5 per cent, biuret had to be fed in suspension. Due to its very steep solubility curve, it was practically impossible to make a homogenous suspension of biuret at the proper feeding temperature. Furthermore, when a concentration of 4 per cent or higher was used, biuret crystallized in the excreted urine at room temperature, clogging various parts of the metabolism cage. Therefore, the results obtained only with lower doses of biuret could be used for the calculation of the dose-action curve. This has the same slope (b = .5507, s = .0597) as that of urea (b = .5631, s = .0814). The mean potency of biuret relative to urea is 1.4.

DISCUSSION. The method described in this paper for assaying diuretics is significantly different from Burn's method for antidiuretics. It has proved to be applicable to the study of all types of diuretics in common use, and gives not only

TABLE 12

Dose-action curves of biuret and its activity relative to urea

DRUG	LOG	LOG % EXCRETION ON							MEAN ACTIVITY
		12-22-41	1-27-42	1-28-42	1-19-43	1-22-43	1-26-43	1-28-43	
Biuret	*mM/kg.*								
	.779	1.611		1.733	1.641	1.666	1.705	1.696	
	.858		1.703	1.714					1.40
	.983		1.814	1.847					
	1.080	1.951	1.889	1.903	1.838	1.780	1.879	1.874	
Urea	1.097				1.706	1.795	1.786	1.837	
	1.319	1.913							1.00
	1.398				1.888	1.909	1.938	2.067	

an accurate estimate of the diuretic potency, but also some information concerning the therapeutic range and the toxic effects of the substance being studied. The limitations of this method, as of most bioassays on animals, arise from the fact that the results of assays on normal animals have to be compared with those on human patients. There is no question that the water-salt balance in the tissues of the normal rat is quite different from that in the tissues of the edematous patient. Nevertheless, it is remarkable that the sequence of activities of known diuretics is almost the same with the rat as it is with the human being: urea, ammonium chloride, potassium acetate, potassium nitrate, theobromine, theophylline, bismuth tartrate and salyrgan (Table 13). Caffeine, due to its very strong side effects, cannot be properly placed in this sequence. The relative potencies of the xanthine diuretics determined on rats, however, are in agreement with those determined by Myers on rabbits (8).

Though active in very small doses, the xanthine diuretics are not capable of

[1] Like urea, biuret will hemolyze erythrocytes in an isotonic solution and is distributed in equal concentration between outer fluid and cell.

producing as intense a diuresis in normal rats as the other substances studied
With high doses of urea or any of the neutral salts, it is possible to obtain within
five hours a urinary excretion approximately twice the amount of the liquid ad-
ministered   Likewise, with salyrgan, a urinary excretion of approximately the
same magnitude may be obtained within the period of its activity   But with the
xanthine diuretics, the amount of urine excreted within five hours is never sig
nificantly greater, and most frequently less than the amount fed   The shape and
the scattering of the points of the dose action curves, especially in the case of
caffeine, suggest that there is a complex mechanism of xanthine diuresis and that a
physiological limiting factor (e g , the antidiuretic hormone) plays an important

TABLE 13

Comparison of diuretic activities from the dose-action curves and from human therapeutic doses

SUBSTANCE	AVERAGE SINGLE HUMAN DOSE			RAT DOSE FOR DIURETIC ACTION 1.5 2.5	DIURETIC ACTIVITY		RATIO RAT DOSE
					Human	Rat	HUMAN DOSE
	gram	*gram/kg*	*ml/kg*	*ml/kg*			
Urea	20–40	0 3–0 6	5–10	12–25	1	1	2 5
Biuret						1 4	
Sodium ace tate						2 0	
Potassium ace tate	15–30	0 23–0 46	2 4–4 8	4–8	2 1	3 4	1 7
Sodium nitrate						2 9	
Potassium ni trate	8–13	0 13–0 20	1 3 2 0	2 5–6 0	4	3 9	2 5
Ammonium chloride	10–20	0 15–0 30	2 8–5 6	4 6–8 9	2	2 7	1 6
		mg/kg					
Theobromine	*0 5–0 6*	*7 7–9 2*	*0 043–0 051*	*0 7–1 4*	*150*	*7 2*	*20*
Caffeine	0 1	1 5	0 008	0 1–0 4	625	abt 32	12 50
Theophylline	0 15–0 20	2 3–3 0	0 013–0 017	0 07–0 16	480	115	5–10
Salyrgan		2 4	0 004–0 008	0 027	1250	abt 400	5
Bismuth Na (K) tartrate	0 03–0 09	0 5–1 5	0 0017–0 005	0 025–0 075	2000–3000	220	15

role, consequently these diuretics have variable effects on different species and
even on different individuals

Urea, biuret and the salts, with the possible exception of ammonium chloride,
showed no toxic effects within the range of doses used   Consequently, the dose
action curves of these substances are linear within this range   The xanthine
diuretics produced definite toxic effects in higher doses   The dose-action curves
of these substances over a wide range of doses, showed some diuresis, and then a
decline   The toxic effects of salyrgan are much more pronounced, and the range
of diuretically active doses is much narrower for salyrgan than for the xanthine
diuretics

The following procedure is recommended for assaying new diuretics   Male rats
weighing 140–240 gm are fasted and deprived of water for 18 hours prior to the

experiment.   Preliminary experiments are done using five groups of 8 rats each on one day.   The control group are fed 25 cc./kgm 0.9% sodium chloride, containing no drug, and the remaining four groups are given geometrically increasing doses of the substance being tested.   Thus the right dose range is determined in very few experiments.   For the estimation of potency, five groups of 8 rats are used on one day.   One group receives no diuretic, two receive two suitable doses of urea (e.g., 0.75 and 1.5 g./kgm.), and the remaining two receive two doses of the substance chosen so that their actions will fall within the same range as those of urea.   The slope, position and the distance between the two regression lines are then calculated.   Three or four such experiments will produce enough data for a sufficiently accurate estimate of the mean activity (potency) relative to urea of the substance.

SUMMARY

1. A method using white rats has been devised for the bioassay of diuretics. It has been tested experimentally and statistically and applied to the study of various substances.   Urea with an activity of 1 was taken as the standard diuretic.

2. The dose-action curves (log dose in millimols per kilogram body weight, plotted against log diuretic action) of urea, various salts, and salyrgan were found to be straight lines of similar slopes within a suitable range of doses. From these the diuretic activities of the substances were calculated.   The dose-action curves of the xanthine derivatives were found to be complex, seemingly limited by a physiological factor.

3. Biuret, a urea derivative, assayed in this way, showed a diuretic activity of 1.4.

4. The following diuretic activities were determined from the dose-action curves: salyrgan, 400; bismuth potassium tartrate, 219; theophylline, 115; caffeine, 32; theobromine, 7.2; potassium nitrate, 3.9; sodium nitrate, 2.9; potassium acetate, 3.4; sodium acetate, 2.0; ammonium chloride, 2.7; and urea, 1.0.

5. Relatively large doses of the salts and urea removed much more water than that which was administered, whereas such doses of the xanthine diuretics, Bi tartrate and of salyrgan not only failed to produce an increase in diuretic action, but in most instances produced a decrease.

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INFLUENCE OF SODIUM BISULFITE ON THE TOXICITY OF EPINEPHRINE

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The following observations resulted from some studies of the pharmacologic properties of suspensions of epinephrine in oil (1), we were, at this time, interested in comparing the toxicity of epinephrine in this form and as an aqueous solution For these investigations, we prepared the aqueous solution in the laboratory from epinephrine powder according to the description of the U S P XII

On one occasion we happened to have no epinephrine powder at hand and used a commercial epinephrine solution instead Such solutions contain 0 1% of epinephrine in the form of the hydrochloride in a dilute hydrochloric acid solu tion and are carefully assayed by the blood pressure method in dogs To our surprise we found that rats died after injection with these solutions at dose levels far below those found with material prepared from epinephrine powder in the laboratory Since the concentration, the amount of HCl used, and the biologic activity as determined by the U S P bioassay method on the blood pressure of dogs with these two types of solutions were identical, it was obvious that another factor had to account for the difference in toxicity It was quickly discovered that the addition of sodium bisulfite, commonly used as an antioxidant for commercial epinephrine solutions, is responsible for this effect The U S P permits the use of sodium bisulfite in concentrations up to 0 5% for such pur poses However, most of the commercial epinephrine preparations contain not more than 0 2% in the 1 1000 epinephrine HCl solutions

A review of the literature (2) showed that sodium bisulfite is relatively non toxic, this was also substantiated by our own experiments Rats tolerated by subcutaneous injections up to 300 mg /kg without gross symptoms The L D 50 was found to be approximately 500 mg /kg We then investigated systemati cally the effect of sodium bisulfite upon epinephrine solutions Rats of either sex, weighing 120–180 gm , were used The animals were not starved before the experiment

The toxicities of epinephrine HCl solution U S P were compared with identical solutions containing 0 1% or 0 2% sodium bisulfite, using different routes of administration The results are summarized in Table I First the lethal doses of plain epinephrine in rats were determined by subcutaneous, intramuscular and intravenous administration using not less than 46 rats for determination of each of the L D s As can be expected, the intravenous lethal dose is considerably lower than the intramuscular or subcutaneous one In fact, epinephrine is 27 times more toxic by intravenous than subcutaneous injection The addition of as little as 0 1% sodium bisulfite to such solutions increased the toxicity by the

subcutaneous route approximately by 100%, and by the intramuscular route approximately three times. If the bisulfite content was raised to 0.2%, still entirely within the limit of the U.S.P., the subcutaneous toxicity was raised more than five times and the intramuscular toxicity about seven times.

A number of experiments were conducted using a 1% concentration of sodium bisulfite with epinephrine which, by subcutaneous injection, brought the toxicity up to one-half of the intravenous lethal dose of a plain epinephrine solution; or, in other words, the addition of 1% sodium bisulfite increased the toxicity by subcutaneous injection 12 times. Both solutions if injected subcutaneously or intramuscularly produced the same symptoms; the animals became restless, cold, and dyspneic; and moderate convulsions appeared. Death occurred after a few minutes to several hours in coma, frequently preceded by pulmonary edema. Signs of intoxication and the fatal outcome were more rapid after intramuscular

TABLE I

Toxicity of epinephrine hydrochloride in rats

(All values are given as mg./kg. of epinephrine base)

0.1% solution

ADDITION	pH	ROUTE OF ADMIN.	APPROX. L.D. 50	NUMBER OF RATS USED
Plain	2.27	subcu.	5.30	96
	2.44			
0.1% sod. bisulfite	2.67			
	2.90	subcu.	2.60	68
0.2% sod. bisulfite	2.62	subcu.	0.97	72
1.0% sod. bisulfite		subcu.	0.43	30
Plain	see above	intramusc.	2.56	95
0.1% sod. bisulfite	see above	intramusc.	0.79	48
0.2% sd. bisulfite	see above	intramusc.	0.37	48
Plain (0.01% sol.)		intravenous	0.20	46
0.2% sod. biulfite		intravenous	0.19	56

than after subcutaneous injection and the addition of sodium bisulfite definitely hastened the course of events in the majority of the animals. The intravenous injection with either solution was immediately followed by severe symptoms.

It is interesting that the intravenous toxicity is influenced very little by the presence of 0.2% sodium bisulfite. This is in accordance with our observation that the blood pressure raising effect of epinephrine in dogs remained also uninfluenced by sodium bisulfite. Before discussing other studies in rats, we should like to give evidence of the presence of this phenomenon in other species of animals.

Table II shows the toxicity of plain epinephrine solutions and those with sodium bisulfite in mice, rabbits, and dogs. We see that a 0.2% concentration of sodium bisulfite again more than doubles the toxicity of plain epinephrine in mice while with as little as 0.02%, a significant increase in toxicity can still be noticed. In rabbits the intramuscular toxicity is more than doubled by the addi-

tion of 0 2% sodium bisulfite In dogs, the epinephrine concentration was raised to 0 2% and the bisulfite concentration correspondingly increased to 0 4% Under these circumstances sodium bisulfite was found to raise the toxicity to more than twice the one of plain epinephrine in a 0 2% solution

It appeared desirable to investigate if the presence of sodium bisulfite would also influence the physiological effects of epinephrine We therefore studied

TABLE II

Toxicity of epinephrine hydrochloride 1 1000 in mice, rabbits, and dogs

SPECIES	ADDITION	ROUTE OF ADMINISTRATION	APPROX LD 50	NUMBER OF ANIMALS USED
			mg /kg	
Mice	Plain	subcu	4 80	30
Mice	0 02% sod bisulfite	subcu	3 60	35
Mice	0 2% sod bisulfite	subcu	2 20	30
Rabbits	Plain	intramusc	1 30	47
Rabbits	0 2% sod bisulfite	intramusc	0 67	30
Dogs	Plain (0 2% sol)	intramusc	4 60	12
Dogs	0 4% sod bisulfite	intramusc	2 00	14
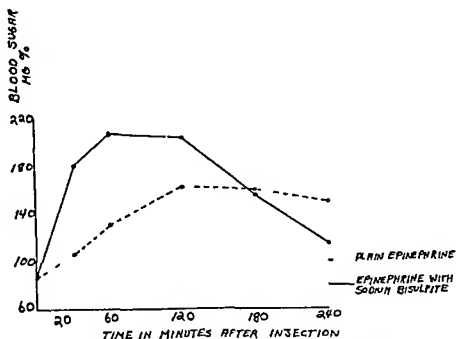
FIG 1 Effect of epinephrine, 0 3 mg /kg , with and without the addition of 0 4% sodium bisulfite upon the blood sugar in dogs after subcutaneous injection

the blood sugar concentration following the subcutaneous injection of 0 3 mg /kg epinephrine in dogs with and without the addition of 0 4% sodium bisulfite Figure 1 shows the results Each point represents the average of blood sugar determinations on 12 dogs It is evident that sodium bisulfite produces a much steeper and considerably higher rise of the blood sugar concentration, which reached its highest point after 60 minutes as compared with 120 minutes with plain epinephrine and tended to drop faster and deeper

This example indicates that it may be desirable for an investigator working on physiological problems concerning epinephrine to contemplate if he wants to use freshly prepared epinephrine solution or a commercial one. Results may be different since the latter almost always contains sodium bisulfite.

DISCUSSION. There are a number of factors which had to be considered in order to explain this potentiating effect of sodium bisulfite. Among these are the acidity and the importance of the presence of a reducing agent. All of our epinephrine solutions were prepared according to the U.S.P. The pH of these solutions being about 2.5 is not influenced by the presence of 0.1 or 0.2% sodium-bisulfite. Neither is their titrable acidity. However, we have prepared a some what more acid plain epinephrine solution by the further addition of 0.5 cc. normal 1/10 HCl acid for each 5 cc. of the U.S.P. solution without an increase in toxicity. Similarly, other reducing agents and other acidifiers were tested which are given in Table III. None of these increased the toxicity of a standard epinephrine solution to any significant extent if used in the concentrations mentioned in Table III. This indicates that the peculiar effect of sodium bisulfite could not be duplicated so far by the addition of other reducing or acidifying

TABLE III

Procedures not influencing resorptive toxicity of epinphrine hydrochloride solution U.S.P.

Addition of 0.1 cc. n/10 HCl per 1 mg. epinephrine
Addition of 10 mg. Ascorbic acid per 1 mg. epinephrine
Addition of 0.1% Hypophosphorous acid
Addition of 0.2% Sodium hypophosphite
Addition of 0.3% Sodium acid phosphate

agents. However, sodium hypophosphite or hypophosphorous acid were found to be satisfactory stabilizers for epinephrine solutions.

It has been shown that epinephrine is readily oxidized in vitro by a number of different enzyme systems; among these the cytochrome system (Green and Richter (3)) and the aminoxidase (Blaschko, Richter, and Schlossmann (4)) appear to be the most important. However, it is very doubtful what role these enzymes play in the biological inactivation of epinephrine in vivo. (Richter, and Tingey (5)); for instance their speed of action has by some been considered too slow to account for the rapid disappearance of epineprine effects in vivo. It is also well known that blood, amino acids, and other tissue constituents tend to protect epineprhine against oxidation. Recently Richter (6) and Richter and MacIntosh (7) have shown that epinephrine is conjugated on one of its phenolic hydroxy groups and excreted as an inactive sulfate ester and these authors assume esterification to be the main mode of biological inactivation. While there is evidence that the liver destroys epinephrine rather rapidly, Markowitz and Mann (8) found no influence of hepatectomy upon the rate of inactivation; but the rat liver decomposes epinephrine rapidly in vitro (9). Clark and Raventos (10) hold that intravenously injected epinephrine diffuses rapidly into the tissues all of which possess the ability to inactivate this substance. Altogether the speed, type, and place of the inactivation are not completely clear as yet.

As already pointed out, the potentiating effect of sodium bisulfite upon epinephrine toxicity is practically absent upon intravenous injection This fact makes a local effect of the sodium bisulfite probable This can be substantiated further experimentally, since injections of 200 mg /kg of sodium bisulfite sub cutaneously followed 15 minutes later by plain epinephrine injections did not result in an increase in toxicity Furthermore, the lethal dose by intramuscular or subcutaneous injection could not be lowered below the order of magnitude of the intravenous dose It appears reasonable, therefore, to assume that the bisulfite enhances the toxicity of epinephrine primarily by a marked local increase in the speed of absorption

Tainter (11) has shown that sodium bisulfite even in weak concentrations pro duces capillary damage and it may possibly be this property which accounts for the faster absorption However, if this is so, it would seem to be a unique effect of sodium bisulfite, since this could not be duplicated by other acid or reducing agents which we have tested to any comparable extent If, beyond this capillary effect, the bisulfite ion influences the inactivating ability of tissue in a specific manner, we are unable to state

We have studied the effect of sodium bisulfite upon a few other drugs (12) The toxicity of ephedrine remained uninfluenced by the addition of this preserva tive, while the L D 50 of methylaminoethanolphenol (Neo Synephrin) by intra muscular injection in rats was reduced to about one half in the presence of 0 2% sodium bisulfite As reported in another paper (13), the toxicity of procaine hydrochloride was found to be increased twice in mice and three times in rats by the addition of 0 1% sodium bisulfite, however, only a slight increase was observed in rabbits and none in dogs

It is well known that the animal detoxifies epinephrine, procaine, and, to a lesser degree, Neo-Synephrin at a rapid rate This is undoubtedly the main reason for the great increment between the subcutaneous and intramuscular toxicity of these drugs on one side and the intravenous toxicity on the other side Epinephrine is, as we have shown, intravenously 27 times more toxic than sub cutaneously As far as procaine is concerned, the subcutaneous toxicity in rats has been reported (14) to be approximately 1 65 gm /kg while the intravenous toxicity is about 50 mg /kg , that is 21 times greater In mice the intravenous toxicity is about 12 times greater than the subcutaneous one, while in the rabbit this ratio is 8 and in the dog only 4 The degree of increase of toxicity produced by the addition of sodium bisulfite is in proportion with these ratios, inasmuch as the most marked increase is observed in rats, somewhat less in mice, little in rabbits and none in dogs

All these observations lend further support to the assumption that the reason for the greater toxicity caused by the addition of sodium bisulfite is an increased speed of absorption which results in a rapid increase of the level of the particular drug in the blood If, however, there is normally no great difference between the intravenous and the subcutaneous or intramuscular toxicity and if the speed of destruction of the drug is not very rapid, no noticeably greater toxicity will result from the addition of sodium bisulfite The failure of sodium bisulfite to

increase the toxicity of ephedrine can be well explained on the basis of the above considerations. It can be expected that drugs having characteristics like procaine and epinephrine with respect to their speed of detoxification, may also experience changes in their toxicity by the addition of sodium bisulfite.

SUMMARY

1. The addition of sodium bisulfite (0.1 to 1.0%) greatly enhances the subcutaneous and intramuscular toxicities of epinephrine HCl solutions in rats, mice, rabbits, and dogs. The intravenous toxicity remains uninfluenced.

2. The increase of epinephrine effects by the addition of sodium bisulfite was also demonstrated in non-toxic doses on the blood sugar curve of dogs.

3. Evidence has been brought forward that this effect is due to a local increase of the speed of absorption produced by sodium bisulfite. A number of other reducing and acidifying agents have been found practically devoid of this action in comparative concentrations. Mention has been made of a similar effect of sodium bisulfite upon a few other drugs.

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COMPARATIVE ACTIVITY OF SULFONAMIDES AGAINST KLEBSIELLA PNEUMONIAE (FRIEDLANDER'S BACILLUS)

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Although acute infections with Friedlander's bacillus as the primary agent do not occur very frequently, their great severity and high mortality rate (1, 2, 3) make it desirable to have available a highly effective therapeutic agent   Clinical reports (4–14) have indicated beneficial effects of sulfanilamide, sulfapyridine and sulfadiazine therapy in Friedlander's bacillus pneumonia and septicemia; however, the number of cases so treated have been too few to permit any conclusions, either as to the value of sulfonamide therapy or as to the relative merits of the different derivatives   Existing experimental studies (15–24) offer little help with these problems.   The most extensive study, that of Feinstone and co-workers (21), has indicated that sulfadiazine has considerable activity against Friedlander's bacillus infections in mice and that its activity is much greater than that of sulfanilamide, sulfapyridine or sulfathiazole.   Feinstone's work is not complete enough to be conclusive, however, for only one dosage of the different drugs was administered and only one strain of Friedlander's bacillus was studied.

The present investigation was carried out in order to obtain more exact information as to the relative activity of various sulfonamides against infections with different strains of Friedlander's bacillus   Sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine, and sulfapyrazine were compared with respect to both *in vivo* and *in vitro* activity.

COMPARATIVE ACTIVITY OF THE SULFONAMIDES AGAINST EXPERIMENTAL INFECTIONS.
Methods   The activity of sulfapyrazine, sulfadiazine, sulfathiazole, sulfapyridine and sulfanilamide was tested against infections with four different strains of Friedlander's bacillus   These strains,[1] two of type A and two of type B, had undergone repeated daily mouse passage   At the time of use their virulence was such that intraperitoneal injection of 1000 to 5000 organisms invariably killed mice within 36 hours

In each test of comparative activity, groups of 170 white mice (males weighing 18 to 22 grams) were used   Each mouse was infected intraperitoneally with 1 cc of a 1 200,000 dilution of a 12 to 14 hour blood broth culture prepared from the heart blood of a passage mouse   As determined by plate counts, this infecting dose contained from 2,000 to 10,000 organisms   Twenty of the 170 mice served as controls, the remainder, divided into groups of 30, were treated with either sulfapyrazine, sulfadiazine, sulfathiazole, sulfapyridine or sulfanilamide[2]   The desired quantity of drug, suspended in 10% acacia, was administered

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117

by stomach tube 2, 8 and 14 hours after infection and at 8-hour intervals thereafter for 5 additional days[3] or as long within that time as the mice survived.

Two groups of experiments were carried out. In the first, the activity of the drugs was compared on the basis of equal dosage, 2.5 mgm. of each of the drugs being administered at the intervals indicated above. In the second group of experiments, activity was compared on the basis of similar concentrations of the drugs in the blood. In this test an attempt was made to obtain sulfonamide levels of 3 to 5 mgm. % at the time midway between treatments, i.e. 4 hours after dosage. The doses of the various drugs required to maintain this level were as follows: sulfapyrazine 2.5 mgm.; sulfadiazine 0.5 mgm.; sulfathiazole 5.0 mgm.; sulfapyridine 2.5 mgm.; sulfanilamide 5.0 mgm. These doses were based on earlier studies (25) on the absorption of these drugs.[4]

RESULTS. *Activities of the sulfonamides administered in equal doses.* From the data in table 1, it is readily apparent that on equal doses of 2.5 mgm., sulfapyrazine and sulfadiazine were definitely more active drugs than sulfathiazole, sulfapyridine and sulfanilamide. This was evident in infections with any of the four strains but was particularly striking in the infection with strain E. Here 100% of the mice treated with sulfadiazine and 77% of those treated with sulfapyrazine recovered, whereas there were only 3% recoveries among the animals receiving sulfathiazole and sulfapyridine and none among those receiving sulfanilamide.

Sulfadiazine and sulfapyrazine were approximately equal in activity. In infections with strains GH, H and E sulfadiazine was slightly more active than sulfapyrazine whereas against strain S sulfapyrazine was somewhat more active.

As to the other three sulfonamides, sulfapyridine was definitely more active than sulfathiazole which in turn was slightly more active than sulfanilamide. None of these three drugs had measurable curative activity against infections with strains GH, H or E. However, in infections with strain S, sulfapyridine cured 80% of the mice as compared with 34% cures with sulfathiazole and 7% cures with sulfanilamide.

It is worth noting that the effectiveness of any one of the drugs varied considerably with the strain of Friedlander's bacillus used as the infecting agent. Thus sulfadiazine, one of the most active drugs, cured only 34% of the animals infected with strain GH, whereas it cured nearly 100% of the mice infected with either strain S or E. Sulfapyridine, one of the weaker drugs, was almost devoid of curative activity against strains GH, H and E, but cured 80% of the animals

[3] Preliminary experiments comparing the therapeutic effects of single doses, treatments for 2 days and treatments for 6 days indicated that the 6-day therapy was the most effective. It was also shown that small doses given for a long period were considerably more effective than large doses given for a short time.

[4] It must be pointed out that the levels of the different drugs were only approximately equal. Differences in the rates of absorption and excretion make it very difficult to maintain the same concentrations of all the drugs throughout the interval between treatments. Sulfathiazole and sulfanilamide are absorbed and excreted more rapidly than the other derivatives so that their maximum levels were somewhat higher and their minimum levels somewhat lower than those of the other sulfonamides. The concentration of sulfapyridine also dropped more than did the concentrations of sulfadiazine and sulfapyrazine. The average levels, taken at the time midway between treatments, were all between 3 and 5 mgm. %. Detailed data on blood levels are given in a previous report (25).

infected with strain S Similar differences were noted among the other drugs In no instance, however, was the relative order of activity of the drugs significantly altered by the use of a particular strain

Activities of the sulfonamides administered in doses that maintain similar concentrations in the blood The results of the experiments in which similar concen-

TABLE 1

The activity of equal doses of sulfapyrazine, sulfadiazine, sulfathiazole, sulfapyridine and sulfanilamide against infections with Friedlander's bacilli

STRAIN	NO OF ORGANISMS IN INFECTING DOSE	NO OF MICE IN GROUP	TREATMENT*	NO OF DEATHS DAYS AFTER INFECTION					AVERAGE HOURS SURVIVAL OF MICE THAT DIED	30-DAY SURVIVORS	
				1 3	4-6	7 10	11 20	21 30		No	Per cent
GH (Type A)	6000	20	None	20	0	0	0	0	27	0	0
		28	SPZ	0	1	13	7	0	246	7	25
		29	SD	0	0	7	12	0	272	10	34
		30	ST	11	17	2	0	0	78	0	0
		30	SP	3	15	12	0	0	133	0	0
		30	SA	27	3	0	0	0	58	0	0
S (Type A)	2900	20	None	20	0	0	0	0	29	0	0
		30	SPZ	0	0	0	0	0		30	100
		28	SD	0	2	1	0	0	141	25	89
		29	ST	0	5	6	4	0	255	10	34
		30	SP	0	2	2	1	1	245	24	80
		30	SA	13	10	5	0	0	95	2	7
H (Type B)	5100	20	None	20	0	0	0	0	23	0	0
		30	SPZ	0	2	21	1	0	211	6	20
		29	SD	0	1	4	7	0	239	17	57
		29	ST	0	29	0	0	0	106	0	0
		30	SP	0	7	23	0	0	154	0	0
		30	SA	16	14	0	0	0	74	0	0
E (Type B)	6200	20	None	20	0	0	0	0	35	0	0
		30	SPZ	0	0	5	2	0	281	23	77
		29	SD	0	0	0	0	0		29	100
		29	ST	11	16	1	0	0	88	1	3
		30	SP	0	12	17	0	0	152	1	3
		30	SA	25	5	0	0	0	57	0	0

* Drugs administered in doses of 2 5 mgm , 2, 8 and 14 hours after infection and every 8 hours thereafter for 5 additional days

SPZ = sulfapyrazine, SD = sulfadiazine, ST = sulfathiazole, SP = sulfapyridine SA = sulfanilamide

trations of the drugs were maintained in the blood are given in table 2 As was true when the doses of the drugs were equal, sulfapyrazine and sulfadiazine had much greater activity than sulfapyridine, sulfathiazole or sulfanilamide In infections with three of the four strains, the activities of sulfapyrazine and sulfadiazine were essentially identical, in infections with the fourth strain (H), sulfapyrazine was more effective than sulfadiazine

It is apparent from comparison of the data in tables 1 and 2 that the activity of sulfadiazine at a dosage of 0.5 mgm. was strikingly similar to its activity on a dosage of 2.5 mgm. In infections with only one strain (H) was the effectiveness of 0.5 mgm. doses considerably less than that of the larger doses.

TABLE 2

The activity of sulfapyrazine, sulfadiazine, sulfathiazole, sulfapyridine and sulfanilamide when average levels of these drugs in the blood were similar

STRAIN	NO. OF ORGANISMS IN INFECTING DOSE	NO. OF MICE IN GROUP	TREATMENT*		NO. OF DEATHS DAYS AFTER INFECTION					AVERAGE HOURS SURVIVAL OF MICE THAT DIED	30-DAY SURVIVORS	
			Drug	Dose	1-3	4-6	7-10	11-20	21-30		No.	Per cent
				mgm.								
GH	4200	20	None		20	0	0	0	0	24	0	0
(Type A)		30	SPZ	2.5	1	1	6	8	0	230	14	47
		30	SD	0.5	0	1	7	10	0	250	12	40
		30	ST	5.0	1	9	19	0	0	150	1	3
		30	SP	2.5	2	19	8	1	0	131	0	0
		30	SA	5.0	12	15	2	0	0	80	·1	3
S	4900	20	None		20	0	0	0	0	26	0	0
(Type A)		30	SPZ	2.5	0	0	0	0	0		30	100
		30	SD	0.5	0	0	0	0	0		30	100
		30	ST	5.0	0	2	6	5	0	246	17	57
		30	SP	2.5	0	3	4	1	0	179	22	73
		30	SA	5.0	9	11	2	0	0	93	8	27
H	10,500	20	None		20	0	0	0	0	24	0	0
(Type B)		30	SPZ	2.5	0	1	15	8	0	229	6	20
		30	SD	0.5	0	0	30	0	0	216	0	0
		30	ST	5.0	1	10	19	0	0	151	0	0
		29	SP	2.5	1	12	16	0	0	143	0	0
		30	SA	5.0	6	23	1	0	0	84	0	0
E	7000	20	None		20	0	0	0	0	22	0	0
(Type B)		30	SPZ	2.5	0	1	2	2	0	217	25	83
		30	SD	0.5	0	2	2	2	0	204	24	80
		30	ST	5.0	0	8	17	2	0	174	3	10
		30	SP	2.5	0	16	11	0	0	142	3	10
		30	SA	5.0	13	17	0	0	0	81	0	0

* Drugs administered in indicated dosages 2, 8 and 14 hours after infection and every 8 hours thereafter for 5 additional days.

SPZ = sulfapyrazine; SD = sulfadiazine; ST = sulfathiazole; SP = sulfapyridine; SA = sulfanilamide.

Of the three less active sulfonamides, sulfapyridine and sulfathiazole had approximately equal activity, the effectiveness of sulfathiazole having risen considerably through increasing the dosage from 2.5 to 5 mgm. On the other hand, a similar increase in the dosage of sulfanilamide produced little increase in activity, this drug being distinctly the least effective of the five sulfonamides studied.

These experiments reemphasize the difference in the effectiveness of the sulfonamides against different strains of Friedlander's bacillus In the test with strain H, only sulfapyrazine possessed curative activity and its administration led to recovery of but 6 of 30 mice In contrast to this, all five drugs had considerable curative activity against strain S, even sulfanilamide leading to the recovery of 8 of the 30 animals

COMPARATIVE ACTIVITY OF THE SULFONAMIDES AGAINST FRIEDLANDER'S BACILLUS IN VITRO Methods The activities of sulfapyrazine sulfadiazine sulfapyridine sulfathiazole and sulfanilamide against Friedlander's bacillus *in vitro* were compared in two different media One medium was a beef heart infusion broth (pH 7 8) containing 2% neopeptone and 0 5% sodium chloride (26), 2% defibrinated rabbit blood was added to this medium just before use The other medium was the synthetic preparation (pH 7 6) of Sahyun and co workers (27) to which was added casein hydrolysate as suggested by MacLeod (28) The casein hydrolysate (SMACO Vitamin Free) was added in a concentration of 0 1%

Concentrated solutions of the various sulfonamides were prepared in each medium These concentrated solutions were diluted with the appropriate basal medium and the dilute solutions were tubed in 9 cc quantities and sterilized by autoclaving After all constituents including inoculum had been added the final sulfonamide concentrations were as follows in the beef heart medium 0 3 0 6 1 25 2 5 5 10 20 40 80 and 160 mgm % sulfapyrazine sulfadiazine sulfathiazole and sulfapyridine and in addition 320 mgm % in the case of sulfanilamide in the synthetic medium 0 02 0 04 0 08 0 15 0 3 0 6 1 25 2 5 and 5 mgm % sulfapyrazine sulfadiazine sulfathiazole and sulfapyridine and 0 3 0 6 1 25 2 5 5 10 20 and 40 mgm % sulfanilamide

In testing the activity of the sulfonamides the following procedure was used The strains of Friedlander's bacillus obtained from a mouse passage culture were subcultured twice in the control medium in which the test was to be performed each subculture being incubated 12 hours A 1 20 000 dilution of the second subculture was prepared in the basal medium and 1 cc quantities of this dilution were added to each of five series of tubes containing the different sulfonamides in the concentrations mentioned above The resulting cultures were incubated at 37 5°C visual measurements of growth being made at 12 24 and 48 hours At least two separate tests were carried out with each of the four strains

RESULTS The results of typical tests are summarized in table 3 From the data presented here it is at once evident that the quantities of sulfonamide required to inhibit growth were much larger in the complex beef heart medium than in the synthetic medium

The relative activities of the various sulfonamides, with the exception of sulfanilamide, also depended upon the medium used in the test Thus in the beef heart medium sulfathiazole was clearly the most effective of the five drugs, being consistently more active than sulfapyrazine, sulfadiazine and sulfapyridine, the latter three compounds were essentially identical in activity In the synthetic medium, on the other hand, sulfathiazole was no more active than sulfapyrazine and sulfadiazine These three sulfonamides were approximately equal in activity and were considerably more active than was sulfapyridine In both media sulfanilamide was by far the least active drug

The amounts of the various drugs required to inhibit growth in the synthetic medium maintained a fairly constant ratio in the case of all four strains As judged by the maximum sulfonamide concentrations permitting growth at 48

hours, sulfapyrazine, sulfadiazine and sulfathiazole were approximately four times as active as sulfapyridine and 60 times as active as sulfanilamide. In the beef heart medium the concentrations of drug required to inhibit growth completely were not always reached. However, where the inhibitory concentrations were attained, sulfathiazole appeared to be about twice as active as sulfapyrazine, sulfadiazine and sulfapyridine and about 30 times as active as sulfanilamide.

The strains of Friedlander's bacillus differed markedly in sulfonamide sensitivity and, with the exception of strain S, the order of sensitivity varied with the test medium. In the beef heart infusion broth, strain E was more sensitive than strain H, which in turn was more sensitive than strain GH. In the synthetic medium, however, strain H was the most sensitive of these three strains and

TABLE 3

Comparison of the IN VITRO *activity of sulfapyrazine, sulfadiazine, sulfathiazole, sulfapyridine and sulfanilamide against Friedlander's bacilli grown in beef heart and synthetic media*

STRAIN	INOCULUM NO. OF ORGANISMS PER CC. CULTURE	HIGHEST SULFONAMIDE* CONCENTRATION PERMITTING VISIBLE GROWTH (MGM. PER CENT)									
		After 12 hours incubation					After 48 hours incubation				
		SPZ	SD	ST	SP	SA	SPZ	SD	ST	SP	SA
Test in beef heart medium											
GH—Type A...	3400	160	160	40	160	320	160	160	80	160	320
S—Type A.....	5100	0.3	0.6	<0.3	0.3	10	0.6	0.6	0.3	0.6	10
E—Type B.....	2700	20	20	5	10	80	80	80	20	80	320
H—Type B.....	2100	20	20	10	20	160	80	80	40	160	320
Test in synthetic medium											
GH—Type A ..	1900	0.02	0.02	0.02	0.08	1.25	0.15	0.15	0.15	0.6	10
S—Type A.....	1000	<0.02	<0.02	<0.02	0.04	0.6	0.04	0.08	0.04	0.3	5
E—Type B.....	1700	0.08	0.08	0.08	0.3	5	0.3	0.6	0.3	1.25	20
H—Type B ...	2100	0.02	0.04	0.04	0.15	1.25	0.08	0.08	0.08	0.6	5

* SPZ = sulfapyrazine; SD = sulfadiazine; ST = sulfathiazole; SP = sulfapyridine; SA = sulfanilamide.

strain E was the least sensitive. In both media strain S was the most sensitive of the four strains. The high sensitivity of this strain may have been due in part to its slow growth. It grew poorly in the synthetic medium and more slowly in the beef heart broth than did the other three strains. Differences in sensitivity of the other three strains were not related to growth since all grew well in both media.

DISCUSSION. The results of the *in vivo* experiments presented in this report suggest that of the sulfonamides tested, sulfadiazine or sulfapyrazine would be the drug of choice in the treatment of Friedlander's bacillus infections. Both dose for dose and on equivalent blood levels, these two sulfonamides were considerably more effective against experimental infections in mice than were sulfapyridine, sulfathiazole or sulfanilamide.

The greater activity of equal doses of sulfadiazine and sulfapyrazine may be partly explained by the absorption and excretion characteristics of these two drugs On the 2 5 mgm doses used in these experiments the concentrations of sulfadiazine in the blood were much higher than those of the other four drugs (25) and these higher levels were maintained throughout the entire period be tween treatments   The maximum concentration of sulfapyrazine produced by this dosage was more similar to the maximum concentrations of sulfapyridine, sulfathiazole and sulfanilamide but unlike these latter three drugs the level of sulfapyrazine was maintained at a constant point throughout the entire interval between treatments

The higher levels of sulfadiazine and the maintenance of a constant amount of sulfapyrazine in the blood are undoubtedly important factors in the superior effectiveness of these two drugs when compared with the others on the basis of equal doses   That these factors do not wholly account for the superior activities of sulfadiazine and sulfapyrazine, however is suggested by the results of experi ments in which the doses of the different sulfonamides were so adjusted that the levels in the blood were more similar   Here again sulfapyrazine and sulfadiazine were much superior to sulfapyridine, sulfathiazole and sulfanilamide   Although it must be admitted that the levels maintained in these experiments were only approximately equal[4], it is believed that the differences in activity were much greater than could be accounted for by these variations alone   It seems prob able, therefore that sulfadiazine and sulfapyrazine have inherently greater activity against infections with Friedlander s bacillus than do the other sulfon amides tested here

It is of interest to compare the order of activity of the various sulfonamides against infections with Friedlander's bacillus with the order of activity against infections with other organisms   The relative activities of various drugs against a specific infection can best be determined when equal levels of the drugs are maintained in the blood   Under such circumstances Long and co workers (29) found that sulfathiazole was more effective against experimental pneumococcal infections than sulfadiazine and sulfapyridine, which were approximately equal in activity   Against infections with *Escherichia coli* White and co workers (30) found that sulfadiazine and sulfathiazole were approximately equal in activity and were somewhat more effective than sulfapyridine and almost ten times as active as sulfanilamide   Long and Bliss (29) found that sulfadiazine and sulfanilamide were equally active against β hemolytic streptococcal infections Our own experiments (25) on β hemolytic streptococcal infections have shown that when similar blood levels are maintained, sulfadiazine sulfapyrazine, sulfathiazole, sulfapyridine and sulfanilamide all possess essentially the same activity   It will be recalled that these orders of activity are all different from that observed above in infections with Friedlander s bacillus, in which sulfadi azine and sulfapyrazine were markedly superior to sulfapyridine and sulfathi azole which in turn were slightly more effective than sulfanilamide   Whether these differences in order of activity indicate that certain drugs have a specific action against certain organisms or whether they merely reflect some peculiar

characteristic of the infection, is not known. In connection with the first suggestion, White and co-workers (30) observed specificity in the *in vitro* action of certain sulfonamides against pneumococci, β hemolytic streptococci and *Streptococcus viridans*. As to the latter suggestion, it is noteworthy that there are important differences in the experimental infections produced by *Escherichia coli*, pneumococci, β hemolytic streptococci and Friedlander's bacilli in the mouse.

A second point brought out in the present study is that infections with different strains of Friedlander's bacillus vary widely in their susceptibility to sulfonamide therapy. This fact not only may account for the conflicting results in the sulfonamide treatment of experimental infections but may well explain the divergent reports and opinions as to the clinical activity of these drugs. It is fairly apparent that infections with some strains of Friedlander's bacillus will respond fairly well to treatment with sulfanilamide while others will not respond well even to sulfadiazine or sulfapyrazine, the most effective drugs.

It was difficult to correlate the results of the *in vitro* tests with the results obtained in the treatment of the experimental infections. In the first place, the order of activity of the drugs was not the same *in vitro* as *in vivo*, except in the case of sulfanilamide, which under both conditions was the least active sulfonamide. *In vitro*, sulfathiazole was as active or more active than sulfadiazine or sulfapyrazine. *In vivo*, however, sulfathiazole was always much less effective than these other two drugs. Sulfapyridine, which *in vitro* was distinctly less active than sulfathiazole, had essentially the same or slightly greater activity *in vivo*. Differences in the order of activity of the sulfonamides *in vivo* and *in vitro* have been noted previously in work on pneumococci (31).

It was also noted that the response of the different strains *in vivo* did not parallel the sensitivity of the organisms *in vitro* (Strain S excepted). This observation is not peculiar to the present experiments but has been noted previously in work with pneumococci (26, 31). Undoubtedly organisms have characteristics such as virulence, invasiveness and antigenicity, which affect the outcome of an *in vivo* experiment but do not affect tests *in vitro*.

In addition to the differences between *in vivo* and *in vitro* activity it was noted that the order of activity of the drugs *in vitro* was affected by the composition of the test medium. In the complex beef heart broth, sulfathiazole was more effective than any of the other drugs against all four strains of Friedlander's bacillus. In the synthetic medium, however, sulfathiazole was no more active than sulfadiazine and sulfapyrazine. In the beef heart broth sulfapyridine appeared to be about as effective as sulfadiazine and sulfapyrazine but in synthetic medium it was considerably less effective. The explanation for this variation in order of activity is not known. Strauss and Finland (32) working with pneumococcus, and White (30) working with *Escherichia coli*, have suggested that the different sulfonamide derivatives are inhibited to varying degrees in different media. White (30) has also suggested that the derivatives may vary in their inhibitory effect on the metabolism of *p*-aminobenzoic acid and peptone. There may be other metabolic processes also which are affected to varying

degrees by the different drugs.   These principles may apply to the effects of the sulfonamides upon the growth of Friedlander's bacillus in different media. , Not only may they explain the differences in activity of the various drugs in the different media used in the *in vitro* tests, but they may also explain the differences found between the orders of activity *in vivo* and *in vitro*.

SUMMARY

A study has been made of the relative effectiveness of sulfapyrazine, sulfadiazine, sulfapyridine, sulfathiazole and sulfanilamide against experimental infections with four different strains of Friedlander's bacillus and against these strains *in vitro*.   Both when equal doses of the different drugs were administered and when similar concentrations were maintained in the blood, sulfapyrazine and sulfadiazine were much more active than sulfapyridine and sulfathiazole which in turn were somewhat more active than sulfanilamide.   Sulfapyrazine and sulfadiazine were approximately equal in activity.

Infections with the different strains varied in their responsiveness to sulfonamide therapy but the order of activity of the various drugs was the same with all strains.

The order of activity of the different drugs *in vitro* was influenced by the composition of the test medium.   In beef heart broth, sulfathiazole was the most effective drug; sulfapyrazine, sulfadiazine and sulfapyridine were approximately equal in activity, and sulfanilamide was the least active of the five drugs.   In the synthetic medium, however, sulfathiazole, sulfadiazine and sulfapyrazine were approximately equal in activity; sulfapyridine was less active and sulfanilamide was again the least active derivative.   Possible explanations for these differences in order of activity are discussed.

The orders of activity of the various derivatives against Friedlander's bacillus *in vitro* showed no correlation with the order of activity *in vivo* except that sulfanilamide was the least active drug in both cases.   The significance of these observations has been discussed.

The various strains of Friedlander's bacillus differed in *in vitro* sensitivity to the sulfonamides.   The orders of sensitivity of the strains were different in the two kinds of media except for strain S, which was the most sensitive strain in both media used.

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ENVIRONMENTAL TEMPERATURE AND DRUG ACTION IN MICE

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In a previous communication (1), it was reported that cymarin and coumingine hydrochloride were much more potent in frogs at high temperatures than at low ones. Using the same specially air conditioned laboratory, we studied 11 additional drugs: diamino diphenyl sulfone, insulin, sulfanilamide, sulfapyridine sodium, sulfathiazole sodium, strychnine sulfate, picrotoxin, tutin, aconitine hydrobromide, harmine hydrochloride, and scopolamine hydrobromide. The specimen of insulin was Standard S 225, 22.5 u. per mg., Insulin Committee, University of Toronto. Both tutin and harmine hydrochloride were isolated in our own laboratory from *Coriaria thymofolia* and *Banisteria Caapi*, respectively (2, 3). The experiments were carried out in white mice of the same strain, each weighing between 14 and 20 gm. With the exception of diamino diphenyl sulfone all drugs were injected intravenously. Solutions of various strengths were prepared, so that the dose volume of each substance by vein did not exceed 0.5 cc. Diamino diphenyl sulfone being practically insoluble in water, was the only member of the series that was used in acacia suspension and given to mice by stomach tube.

The special laboratory employed in the work measures $332\frac{1}{2}$ square feet, and $10\frac{1}{4}$ feet high. The range of temperature possible in the room is from 25 to 40°C dry bulb all year round with a fluctuation of ± 0.25°C., having a relative humidity of 40 per cent and an almost constant air current. The desired conditions are controlled by a set of wet- and dry bulb thermostats [1]. In the winter months when the outside air is cool, a temperature of 20°C or less dry bulb in the room can be attained. In that case the air current and relative humidity have to be altered. The animal cages, resting on racks were placed in the center of the room, and work benches installed next to one of the walls. Prior to administration of drugs, all mice were starved for 16 to 18 hours.

The object of the experiment was to determine at each temperature the toxicity of 10 substances by employing several dose levels and arriving at the median lethal dose (LD$_{50}$). With insulin, strychnine sulfate, picrotoxin, and tutin, the median convulsive dose (CD$_{50}$) was carefully estimated. The LD$_{50}$ of the last 3 products was also ascertained. The number of mice used for the determination of either LD$_{50}$ or CD$_{50}$ varied from 9 to 145. The number of animals on each dose also varied—a minimum of 4 and a maximum of 20.

The results are summarized in table 1. The median lethal doses (LD$_{50}$ ± standard error) of diamino diphenyl sulfone, sulfanilamide, sulfathiazole sodium, strychnine sulfate, tutin, aconitine hydrobromide, harmine hydrochloride, and

TABLE 1

Room temperature and drug reaction in mice

DRUG	REACTION STUDIED	ROOM TEMPERA- TURE	NUMBER OF MICE USED	MEDIAN DOSE ± S.E.	
		°C.		*mg. per kg.*	
Diamino-diphenyl sulfone	LD_{50}	20	65	382.3	±43.8
		25	45	495.8	±53.2
		30	75	255.0	±22.2
		35	80	151.5	±15.0
		40	145	49.82	± 3.26
Insulin	CD_{50}	20	51	> 0.009	
		25	40	0.005644 ± 0.001210	
		30	78	0.001630 ± 0.000206	
		35	40	0.000311 ± 0.000060	
		40	50	0.000107 ± 0.000017	
Sulfanilamide	LD_{50}	20	35	636.5	±15.4
		25	9	621.0	±32.6
		30	20	557.7	±16.8
		35	15	631.1	±23.0
		40	20	623.0	±22.3
Sulfapyridine sodium	LD_{50}	20	25	758.2	±22.2
		25	40	846.5	±19.0
		30	25	837.9	±16.1
		35	30	742.3	±57.4
		40	40	481.2	±15.0
Sulfathiazole sodium	LD_{50}	20	30	969.0	±44.7
		25	40	1244.0	±50.0
		30	40	1013.0	±35.0
		35	30	902.8	±36.5
		40	40	845.0	±28.9
Strychnine sulfate	CD_{50}	20	30	0.3682	± 0.0075
		25	45	0.3751	± 0.0137
		30	35	0.3060	± 0.0097
		35	30	0.4042	± 0.0140
		40	55	0.3655	± 0.0232
Strychnine sulfate	LD_{50}	20	55	0.4296	± 0.0172
		25	45	0.4754	± 0.0271
		30	50	0.4041	± 0.0165
		35	35	0.4192	± 0.0205
		40	45	0.3580	± 0.0151
Picrotoxin	CD_{50}	20	25	1.585	± 0.079
		25	15	1.530	± 0.092
		30	25	1.298	± 0.059
		35	15	1.581	± 0.125
		40	20	1.140	± 0.068

TABLE 1—Continued

DRUG	REACTION STUDIED	ROOM TEMPERATURE	NUMBER OF MICE USED	MEDIAN DOSE ± S E	
		°C		mg per kg	
Picrotoxin	LD$_{50}$	20	35	3 454	± 0 094
		25	45	2 895	± 0 090
		30	50	2 682	± 0 084
		35	15	2 531	± 0 234
		40	30	1 958	± 0 229
Tutin	CD$_{50}$	20	20	1 266	± 0 076
		25	20	1 320	± 0 043
		30	40	1 060	± 0 049
		35	15	0 968	± 0 030
		40	15	0 941	± 0 030
Tutin	LD$_{50}$	20	40	2 423	± 0 073
		25	30	2 990	± 0 131
		30	25	2 685	± 0 099
		35	25	2 073	± 0 095
		40	25	1 774	± 0 070
Aconitine hydrobromide	LD$_{50}$	20	35	0 2891	± 0 0158
		25	40	0 2321	± 0 0116
		30	45	0 1875	± 0 0102
		35	50	0 2051	± 0 0097
		40	30	0 1719	± 0 0106
Harmine hydrochloride	LD$_{50}$	20	34	44 67	± 2 87
		25	40	38 19	± 1 89
		30	40	35 15	± 1 76
		35	25	32 53	± 2 06
		40	50	21 66	± 1 17
Scopolamine hydrobromide	LD$_{50}$	20	40	190 4	± 8 7
		25	30	202 5	±12 0
		30	23	218 1	±19 6
		35	20	210 8	±16 8
		40	25	153 5	±12 0

scopolamine hydrobromide, and the median convulsive doses (CD$_{50}$ ± standard error) of picrotoxin and tutin were computed by the combined slope of all temperatures—as shown in the last column With the exception of 20°C, the median convulsive doses of insulin were also computed by the common slope The median lethal doses of sulfapyridine sodium and picrotoxin, and the median convulsive doses of strychnine sulfate, on the other hand, were calculated from their individual slopes, for the χ² of the combined slope exceeded the 5 per cent point (4)

It is obvious that temperature has a remarkable effect upon the toxicity of diamino diphenyl sulfone At 40°C the substance is almost 10 times as toxic

as at 25°C. There is a slightly higher toxicity at 20°C. than at 25°C., but no adequate explanation can be offered at present for this irregularity.

The temperature effect with insulin in mice by intravenous injection is even more impressive. At 20°C. only 6 out of 51 mice convulsed, covering a very wide range of doses, from 0.001 to 0.009 mg. per gm. The median convulsive dose (CD_{50}), if estimable, must therefore exceed 0.009 mg. per gm. The average time before the onset of convulsions in the 6 mice was 115 ± 30 minutes. When the temperature was raised to 25, 30, 35, and 40°C., the CD_{50}'s became progressively smaller, indicating an increase in potency with the rise of room temperature. Indeed, it may be said that insulin in mice at 40°C. is more than 50 and 80 times as potent as at 25 and 20°C., respectively. The speed of action also increases with the rise of room temperature as judged by the decrease of the latent period for development of convulsions. The average time between injection and first convulsion was 156 ± 19 minutes at 25°C.; 95 ± 7, at 30°C.; 41 ± 4, at 35°C.; and 28 ± 3, at 40°C. The above data substantiate the conclusions of our predecessors. Voegtlin and his associates (5, 6) stated that the toxicity of insulin in rats increased with the rise of room temperature. Huxley and Fulton (7) showed that the speed of action of insulin in frogs also increased with the elevation of temperature. Other investigators (8–10) reported that convulsions following insulin, in the cat, the dog, the woodchuck, and the mouse, were inhibited by a drop of atmospheric temperature.

Of the 3 sulfonamides, sulfapyridine sodium and sulfathiazole sodium appear to show a similar trend in toxicity, but much less decisive as compared with diamino-diphenyl sulfone and insulin. The toxicity of sulfanilamide is the only one that is not influenced by changes of temperature. Larson, Levine, Bieter, and McLimans (11) found that mice infected with Type II pneumococci and treated with sulfanilamide lived longer at 21°C. than at 36.7°C. No information is available as to whether or not the remaining chemotherapeutic agents of our series have a similar behavior.

The convulsive dose of strychnine sulfate in mice is apparently unaltered by temperature. Convulsions took place so quickly that practically no difference in the rapidity of onset of convulsions could be detected at various temperatures. The same can be said of its lethal effect. Only at 40°C. is the toxicity of this alkaloid slightly elevated as shown by the smaller LD_{50}. In this connection, it would be interesting to mention the data for frogs and toads—both cold-blooded animals—published by other workers. Schlomovitz and his associates (12, 13) demonstrated in frogs that the average time between the injection of strychnine and the first convulsion was 565 seconds at 2°C., and 34 seconds at 36°C. In fact the response was so uniform that the authors could predict the onset of convulsions at any temperature. Lambruschini (14) found that in the South American toad, *Bufo arenarum*, the fatal dose of strychnine sulfate at 3°C. was 0.15 mg. per gm. and that at 30°C., 0.06 mg. per gm. The drug in both species of animals was of course administered by injection into the lymph sac, while in our experiments with mice, it was given intravenously. The results are, therefore, not entirely comparable

The response of mice to picrotoxin in developing convulsions is somewhat

irregular at different temperatures—the CD_{50} being smallest at 40°C, next at 30°C, and still next at 35, 25, and 20°C In general, as shown in table 1, the doses are not very far apart There is some evidence of difference in the speed of action The average time between injection and first convulsion was 17 4 ± 1 8 minutes at 20°C, 18 ± 1 5, 25°C, 12 1 ± 1 6, 30°C, 10 ± 2 9, 35°C, and 10 3 ± 0 8, 40°C The median lethal doses vary more uniformly with temperature, smallest at 40°C and largest at 20°C The response of the mouse is again not the same as that of the frog for Fuhner and Breipohl (15) reported that picrotoxin was 3 times as toxic to frogs at 30°C as at 19°C

With tutin the environmental temperature has some effect on its convulsive dose, being smallest at 40°C There is a discernible difference in the speed of action between two temperature ranges, namely, 20–25°C and 30–40°C The average time between injection and first convulsion is as follows 30 9 ± 3 2 minutes at 20°C, 28 5 ± 3 2, 25°C, 20 8 ± 1 3, 30°C, 19 9 ± 1 2, 35°C, and 21 4 ± 1 6, 40°C The toxicity of tutin as judged by its LD_{50} follows a similar trend At 40°C, it was relatively more toxic than at lower temperatures Aconitine hydrobromide has a tendency to be less toxic at 20–25°C than at 30–40°C The increase in the toxicity of harmine hydrochloride with the rise of temperature is regular and definite At 40°C, it is slightly more than twice as toxic as at 20°C Regarding scopolamine hydrobromide, it is significantly more toxic at 40°C than at 20–35°C

The rectal temperature of all the insulin mice was recorded by a thermocouple before injection The average rectal temperature was 34 65° ± 0 25°C at room temperature of 20°C, 35 04° ± 0 19°C, at 25°C, 36 83° ± 0 13°C, at 30°C, 37 86° ± 0 16°C, at 35°C, and 38 48° ± 0 10°C, at 40°C These fluctuations are in line with Herrington s findings (16) It appears that in some instances a pharmacologic reaction has a resemblance to a chemical reaction Thus, in the present series of experiments, a rise in environmental temperature, which is responsible for a rise in the mouse's rectal temperature, increases both the speed and intensity of insulin action on the one hand, and the toxicity of diamino diphenyl sulfone on the other This, however, is not true with all drugs, for the action of some is almost independent of temperature Equally important is of course the animal employed Certain animals show a marked variation of susceptibility to drugs due to temperature, while others are able to withstand such changes without altering their response to drugs

<div align="center">SUMMARY</div>

1 Of 11 drugs studied in mice at various temperatures, diamino diphenyl sulfone given by mouth is shown to be approximately 10 times as toxic at 40°C as at 25°C

2 Insulin injected by vein is more than 80 times as potent at 40°C as at 20°C The onset of convulsions occurs sooner at high temperatures than at low ones

3 Harmine hydrochloride administered intravenously is also increasingly toxic, although to a less degree, with the rise of temperature It is about twice as toxic at 40°C as at 20°C

4 There is a suggestion that the toxicity of sulfapyridine sodium, sulfathia

zole sodium, strychnine sulfate, picrotoxin, tutin, aconitine hydrobromide, and scopolamine hydrobromide, increases with the elevation of temperature—particularly at 40°C. as compared with lower temperatures. The lethal dose of sulfanilamide is least influenced by temperature.

5. Mice appear more susceptible at 40°C. to picrotoxin and tutin in developing convulsions than at 20–25°C. The speed of action is also accelerated at higher temperature levels.

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THE ACTION OF CARTHAMOIDINE

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Alkaloids obtained from various species of *Senecio* are of particular interest, because the majority of them so far studied produce hepatic necrosis when ad ministered to laboratory animals (1–6) For some time Adams and his co workers (7–14) have been investigating the structure of monocrotaline, an alkaloid similar, both chemically and pharmacologically (15), to *Senecio* bases Their work has been extended to several new alkaloids of *Senecio* (16) In June, 1941, Professor Roger Adams, department of chemistry, University of Illinois, Urbana, generously supplied us with a specimen of carthamoidine, an alkaloid isolated in his laboratory from *Senecio carthamoides* On account of the war, his chemical work has been interrupted, and the publication of his results is consequently postponed However, Professor Adams has kindly permitted us to report our data in animals at this time

A 1 percent solution of carthamoidine was prepared by dissolving the material in an equimolecular amount of hydrochloric acid Single, graded doses were injected intravenously into albino mice A total of 50 animals was employed Death was gradual, occurring in from a few hours to 3 days In general, the mice which received the largest doses died first The observation period was 1 week, at the end of which all the surviving mice were chloroformed The median lethal dose ± standard error, computed from the mortality figures, as given in table 1, was $68\,32 \pm 2\,44$ mg per kg

Necropsies were carried out on 23 mice that died, and on 23 others that were sacrificed Of the former group, 1 animal injected with a dose of 62 mg per kg died 7 days after injection, obviously due to an infection Its viscera showed no lesions attributable to the drug Of the remaining 22 mice, 5 developed slight ascites, 1, slight hydrothorax, and 1, pulmonary edema Four of 13 thymuses examined showed necrosis of cortical lymphocytes

As with the administration of other *Senecio* alkaloids, the most prominent feature following the injection of carthamoidine was necrosis of the liver Necrosis was periportal in 18 mice, midzonal in 3 others, and central in only 1 animal An example of periportal necrosis is illustrated in figure 1 The areas of necrosis are therefore more like those caused by pterophine—namely, predominantly periportal (6) With the remaining alkaloids of *Senecio* which we have studied, central necrosis of the liver is the usual picture

Associated with necrosis were sinusoidal congestion of various degrees, and hemorrhage into the cords of necrotic cells In the livers of 8 mice, congestion and hemorrhage were so localized and intense that a resemblance to cavernous hemangioma occurred a lesion we have previously observed with other *Senecio* alkaloids In some regions, hemorrhage was so severe that cell cords were dis

rupted, and it appeared that many cells had been washed away.   Leukocytic infiltration of necrotic cells was usually slight, and was never abundant.   One of

TABLE 1
Toxicity of carthamoidine

| DOSE | NUMBER DIED NUMBER USED | NUMBER AUTOPSIED | |
		After death	After sacrifice
mg. per kg.			
50	1/5	1	4
56	0/5		5
62	2/10	1*	8
70	4/10	4	6
80	10/10	10	
100	5/5	4	
125	5/5	3	

* This animal died 7 days after injection from an intercurrent infection.
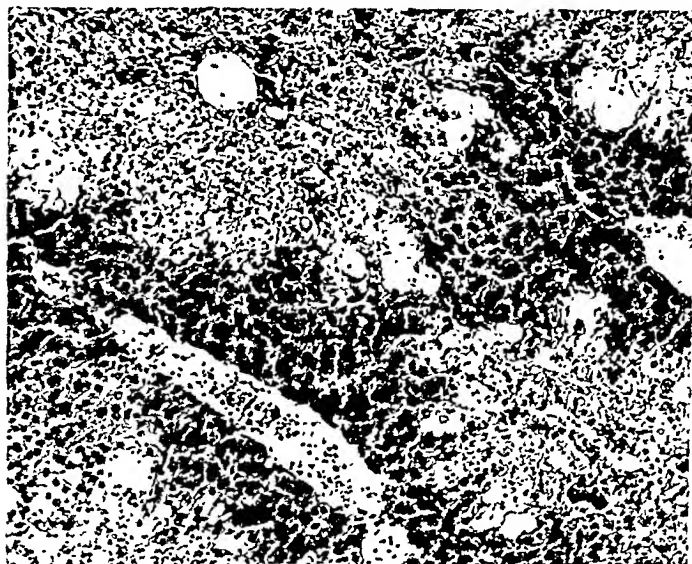
Fig. 1. Periportal necrosis of the liver produced by carthamoidine.   × 116. Mouse, numbered 35, female, received a single intravenous dose of 70 mg. per kg. of carthamoidine and died 30 hours later.   There is extensive periportal necrosis with hemorrhage into cords of necrotic cells, and in several places there is extreme sinusoidal congestion or hemorrhage. Erythrocytes are not clearly seen.   The small irregular black dots are nuclei of leukocytes and fragments of liver cell nuclei.   A small bile duct is visible next to one of the two portal veins in the field.   Cells about the central veins are normal.

the mice which showed midzonal hepatic necrosis also had acute intracapillary glomerulonephritis.   This is the only example of renal injury out of the 50

animals injected   It may not be necessarily due to the toxic action of the alkaloid   On the other hand, seneciphylline uniformly induced cloudy swelling of the kidney, as shown previously in this laboratory (4)

In the 23 mice which survived doses ranging from 50 to 70 mg per kg , no gross abnormalities were observed   Tissues of 5 were examined microscopically, and found normal

Carthamoidine inhibited the isolated rabbit's small intestines in concentrations of 1.10,000 to 1 5,000   It stimulated the isolated guinea pig's uterus in the concentration of 1.10,000   In etherized cats, 50 mg of carthamoidine injected by the femoral vein caused a marked fall of arterial blood pressure with prompt recovery.

SUMMARY

Carthamoidine in suitable doses produces slow death and necrosis of the liver in mice when injected intravenously   The lesion is predominantly periportal, similar to that caused by pterophine   Carthamoidine inhibits the isolated rabbit's intestines, stimulates the isolated guinea pig's uterus, and lowers blood pressure in etherized cats

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THE EFFECT OF URINARÝ pH ON THE ELIMINATION OF QUININE IN MAN

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Recently it was reported from this laboratory that in human smokers urinary nicotine elimination is markedly affected by the pH of the urine (1). In brief, it was found that when the urine was alkaline only one-fourth as much nicotine was eliminated as when the urine was acid. This difference was explained on the basis of greater nicotine resorption through the urinary mucosa from an alkaline urine, the alkalinity increasing the proportion of alkaloidal base, the form in which, presumably, the alkaloid is most efficiently resorbed.

Because the pK_a value of quinine is so similar to that of nicotine (8.3 vs. 8.07, fig. 1), it was thought that the elimination of quinine might behave in a manner similar to that of nicotine. This possibility seems especially interesting in view of the observations of Gnilosirova-Guryea (2) on the importance of alkaline diets during quinine therapy, and Sinton's (3) recommendation of alkalies as useful adjuvants to quinine in the treatment of malaria. Accordingly, we have made the following studies on the effect of changes in urinary pH on the urinary elimination of quinine in man.

EXPERIMENTAL. The subjects were male medical students in apparent good health. No restrictions were placed on their habits, except that they were requested to keep their urine volume as constant as possible during the experimental periods.

For the purpose of acidifying the urine 1 gram of ammonium chloride was given orally four times a day; for alkalizing the urine 4 grams, more or less, of an effervescent alkaline preparation was given six times a day. No difficulty was encountered in maintaining the urine acid. With some individuals alkalinization was difficult, and with one subject it was impossible to maintain the pH above 6.94 satisfactorily. pH determinations were made with a glass electrode.

One-half gram quinine (as the sulfate) was administered orally in capsules when the urine had reached the desired pH, and two consecutive 24 hour samples of urine were collected. The subjects continued to take the acidifying or alkalizing drugs during the entire period. In some subjects a 24 hour control specimen of the urine was also collected immediately before administration of the quinine.

Quinine determinations were made by the fluorimetric method of Kelsey and Geiling (4) on ether extracts of the urine. In three experiments in which 10 mg. of quinine was added to 100 cc. of urine the average recovery was 98%. Specimens of urine from four subjects prior to receiving quinine were entirely negative for quinine by this method.

A second series of quinine determinations was made on the urine without extraction and purification. Control experiments showed that if properly diluted (1:100–1:200) the average recovery amounted to 92% of the quinine added. When four samples of urine from subjects not taking quinine were tested by this technic fluorimetric readings were obtained, indicating the presence of from 0 to 4 mg. equivalent of quinine for a 24 hour sample.

RESULTS AND DISCUSSION. Table 1 gives the results obtained on ether extracts of urine, and shows that about twice as much quinine was eliminated

when the urine was acid as when the urine was alkaline   This indicates to us
that quinine is more actively resorbed from the urinary tract when the urine is
alkaline than when it is acid   The elimination values as a whole fall within the
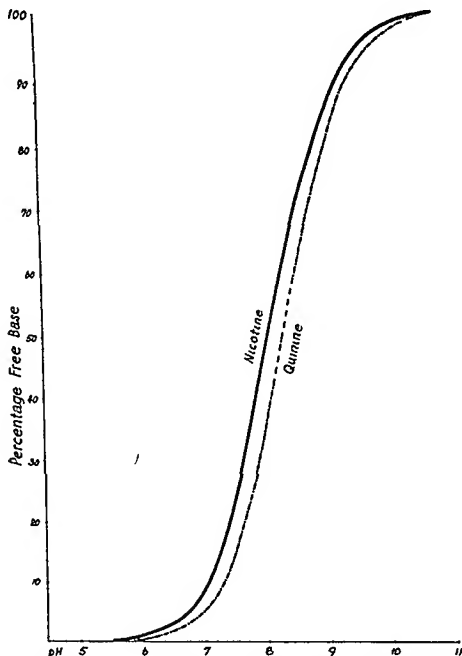
FIG 1 EFFECT OF pH ON THE PERCENTAGE OF NICOTINE AND QUININE PRESENT AS THE
ALKALOIDAL BASE

The above curves were constructed from values obtained using the formula $pH = pK_a +$
$\log \frac{base}{salt}$ The pK_a value used for quinine was that obtained by Sir S Rickard Christophers
(Ann Trop Med **31** 43 1937)   The pK_a value used for nicotine was obtained by us by
electrometric titration

range reported by other investigators (5)   Subject No 10 was unable to elevate
his urinary pH above 6 94, nevertheless there was a slight difference between
the quinine elimination at this pH and at pH 5 06

While it has been shown that the oral administration of alkalies apparently facilitates absorption of quinine from the gastrointestinal tract (6), this in itself could not account for a decreased urinary elimination; rather one would expect the reverse.

We believe that the reason relatively greater resorption from the urinary tract occurs with nicotine in the case of smokers than with quinine in the present experiments is due largely to two factors. First, there is a difference between the pK_a values of nicotine and quinine which may be favorable to greater resorption of nicotine. Secondly, in the case of the quinine administration, the drug was given in one single large dose, whereas, with nicotine, its intake, and hence

TABLE 1

Percentage of ingested quinine in ether extracts of urine

SUBJECT	ALKALINE URINE							ACID URINE						
	First 24-hour sample			Second 24-hour sample				First 24-hour sample			Second 24-hour sample			
	pH	Volume	Amount excreted	pH	Volume	Amount excreted	Total amount excreted	pH	Volume	Amount excreted	pH	Volume	Amount excreted	Total amount excreted
		cc.	per cent		cc.	per cent	per cent		cc.	per cent		cc.	per cent	per cent
1	7.20	2,140	9.4	7.22	2,380	1.5	10.9	5.80	2,100	14.0	5.38	1,200	1.9	15.9
2	7.47	1,535	6.5	7.61	1,300	1.6	8.1	5.48	1,915	18.9	5.19	1,660	2.6	21.5
3	7.72	2,350	9.2	7.65	1,720	1.1	10.3	5.17	3,115	13.7	5.21	1,540	1.4	15.1
4	7.47	1,315	4.5	7.21	1,735	0.7	5.2	5.19	1,665	13.2	4.99	1,515	1.5	14.7
5	7.69	1,835	5.9	7.37	1,415	0.9	6.8	5.09	3,935	10.1	5.19	2,120	3.4	13.5
6	7.30	1,620	7.4	7.62	1,025	1.0	8.4	5.05	1,610	19.5	5.14	1,275	3.0	22.5
7	7.60	1,675	9.3	7.80	1,825	1.7	11.0	5.09	1,900	15.3	5.13	1,830	1.9	17.2
8	7.38	2,065	8.7	7.59	1,570	1.9	10.6	5.02	1,890	13.6	4.94	1,073	1.1	14.7
9	7.57	1,250	6.2	7.60	1,165	2.7	8.9	5.14	925	17.7	4.97	755	3.4	21.1
Averages	7.49	1,754	7.45	7.52	1,570	1.45	8.9	5.22	2,117	15.1	5.12	1,441	2.25	17.4
10*	6.94	1,485	9.8	6.21	790	2.7	12.5	5.21	1,500	12.0	5.06	872	2.1	14.1

* See text.

its elimination, in the case of smokers, was gradual during a period of many hours.

On averaging the 48 hour values obtained on unextracted urine the fluorescent equivalent of 25.7% of the administered quinine was found in the urine when it was maintained alkaline, and 29.2% when it was maintained acid. Comparison of these figures with those gotten on extracted urine indicates that after quinine administration there occurs in the urine a fluorescent quinine metabolic product (or products) that is not extractable with ether. In view of this it may be well to reserve judgment as to the exact identity of the fluorescent material in the ether soluble fraction of such urine. At present, the possibility that the latter fraction may include ether soluble fluorescent metabolic products of quinine in addition to unchanged quinine has not been eliminated. However, due to the lack of suitable methods of proven specificity for the determination of

quinine in biological materials we have tentatively assumed that the ether soluble fraction represents unchanged quinine

While the subjects of these tests were requested to keep their urinary volume reasonably constant during the experimental periods, they did not always do this too well However, comparison of the total urinary volumes (48 hr) of the nine subjects while the urine was maintained alkaline and while it was maintained acid, showed no statistically significant differences Further evidence that variations in urinary volume of the magnitude encountered was not an important deciding factor in these excretion studies may be adduced from the results obtained in 2 subjects in whom the urinary volume was deliberately varied (table 2)

TABLE 2

Relationship between urinary volume and quinine excretion following the oral administration of 600 mg quinine

SUBJECT	LOW URINE VOLUME			HIGH URINE VOLUME		
	pH	Urine	Quinine eliminated first 24 hours	pH	Urine	Quinine elm nated fi st 24 hours
		cc	*mg*		*cc*	*mg*
1	5 80	2 100	70	5 45	4 290	53 9
11	5 15	1 030	65	5 50	3 870	69 7

SUMMARY

When quinine was given in a dose of 500 mg orally to human subjects it was found that the extent of urinary elimination was related to the pH of the urine When the urine was maintained alkaline one half as much quinine, on an average, was eliminated as when the urine was acid (8 9 vs 17 4%) This difference is assumed to be due to greater resorption of quinine from the urinary tract when the urine is alkaline than when the urine is acid

Although the amount of quinine ' saved" to the patient is small on alkalinization of the urine, this saving plus the advantages of an alkaline regime suggested by clinical investigators on the basis of other types of observations, seem to recommend further consideration of the use of alkalies in the quinine therapy of malaria

These experiments again indicate the importance of considering the possible influence of urinary pH in quantitative studies on the urinary excretion of certain chemicals

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PHYSICO-CHEMICAL PROPERTIES OF THE ARSPHENAMINES IN RELATION TO DISTRIBUTION AND RETENTION IN THE TISSUES[1]

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Toxic reactions to the arsphenamines have been reported since shortly after the introduction of arsphenamine by Ehrlich (7) in 1909. These toxic reactions may be (1) acute reactions occurring shortly after the administration of the drug, (2) delayed reactions occurring one to seven days after administration or (3) reactions resulting from the accumulation of the drug in the body.

The causes of toxic reactions of the first two types have been investigated by a number of workers. At first it was thought that these untoward reactions were due in part to the presence of 3-amino-4-hydroxy phenyl arsenious oxide (arsenoxide) (7, 8, 9), but subsequently the conviction has grown that the toxic reactions were closely related to the physical characteristics of both arsphenamine and neoarsphenamine (15, 17, 29, 32, 33). Thus it was shown that there was a close parallelism between the viscosity and toxicity of different samples of these drugs and that the method of manufacture influenced the degree of viscosity (19, 34, 39). The history of this period has been reviewed in detail by Wright et al. (45).

The separation of arsphenamine and neoarsphenamine into a crystalloid and colloid fraction was attempted by Raiziss and Gavron (28) using parchment membranes which are relatively impermeable, and was later expanded by Wright et al. (45), who used viscose membranes which are more permeable. The latter investigators studied the therapeutic and toxic properties of the two fractions after separation by dialysis and found that the crystalloid fraction (1) was more curative for trypanosomiasis in rats (2) was less toxic and (3) produced death only after the administration of large doses and followed the typical symptoms of arsenical poisoning, while the colloid fraction produced immediate toxic reactions resulting in death from respiratory failure with comparatively small doses.

These reactions produced by the colloidal fraction were of essentially the same type as the nitritoid reactions occurring in human beings, and it appeared probable that nitritoid reactions in human beings were due to the colloidal properties possessed by the arsphenamines. It is particularly significant that mapharsen, which is crystalloid in nature, has not produced a single proven nitritoid reaction in more than twelve million injections (23). Wright et al. (45) concluded that

the desirable properties of a high curative index and a low toxic index appeared to reside in the least aggregated portion of the arsphenamines, and that the strongly aggregated colloidal portion appeared to possess only the undesirable properties of low curative index and a high toxic index, producing immediate toxic reactions after injection in small dosage. Morell and Allmark (25) in an investigation of the variations in toxicity and trypanocidal activity of commercial specimens of neoarsphenamine were of the opinion that brands with a low toxicity showed a high trypanocidal activity and also that those which were most toxic were usually but not invariably the least active. This would appear to substantiate the findings of Wright et al. (45).

In view of the marked difference in both therapeutic and toxic properties of the crystalloid and colloid fractions of the arsphenamines, it appeared desirable to investigate possible differences in the tissue affinity and storage of these separated fractions, since this might have an important bearing upon the question of cumulative poisoning.

It is more or less established that following the injection of either arsphenamine or neoarsphenamine considerable quantities of arsenic remain in the body for periods of longer than fourteen days (3, 14, 20, 21). Since the therapeutic activity of the arsphenamines appears to be dependent upon conversion in the body to arsenoxide (6, 31) and since Wright et al. (43, 46) have found that following the direct injection of arsenoxide (mapharsen), there appears to be appreciably less cumulation of arsenic in the tissues, it might be deduced that that portion of arsphenamine or neoarsphenamine which is converted into arsenoxide in the body would be likely to be more rapidly excreted. This would suggest the further possibility that that portion of arsphenamine and neoarsphenamine which is rapidly converted into arsenoxide following the injection of these drugs might be the crystalloid fraction, while that portion of the arsenical which becomes relatively firmly fixed by the tissues might be the colloidal portion. Hence it was decided to investigate the manner in which the separated crystalloid and colloid fractions of arsphenamine and neoarsphenamine distributed themselves to the various tissues of the body in comparison with that of the whole drug, and to investigate the length of time which the arsenicals, both as whole drug and as separate fractions remained in those tissues.

The present knowledge of the distribution of organic arsenicals in the body is for the most part incomplete, since most investigators have accounted for only a small part of the injected arsenic. Wilcox and Webster (42), in 1916 found that arsenic was present for two to three weeks after the injection of arsphenamine. Hopper, Kolle and K. D. Wright (16), in 1921, showed by histological examination that arsphenamine has an affinity for the liver. Voegtlin and Thompson (41), in 1922, found that both arsphenamine and neoarsphenamine left the blood stream rapidly and were fixed by the tissues, especially by the liver and spleen, in a more or less firm combination. Clausen and Jeans (3), also in 1922, found after the intravenous administration of arsphenamine in children that the concentration of the drug in the blood stream dropped rapidly until it contained only 10.5% of the injected drug at the end of one hour, and only 1.2% after twenty-

four hours. They also studied the distribution of arsphenamine in kittens following intravenous administration and found that the liver, small and large intestine, spleen and kidneys took up most of the arsenic. The sum of their recoveries of arsenic at the end of twenty-four hours amounted to 12.5% of the injected dose.

Kolls and Youmans (20), in 1923, found that about three-fourths of the administered arsphenamine had left the blood stream a few minutes after completion of the injection and that the drug was stored in the liver, spleen, kidneys, lungs and in cardiac and skeletal muscles. Bulmer (2), in 1923, found a high concentration in the liver shortly after injection, but that later this concentration became much smaller. He found a relatively large amount in the lungs, which was maintained over a period of at least several days. He also concluded that arsenic was retained by the long bones over a longer period than was shown by analyses of any other tissues. Voegtlin, Smith, Dyer and Thompson (40) found that arsphenamine and neoarsphenamine penetrated the spinal fluid with considerable difficulty.

Fordyce, Rosen and Myers (10), in 1922, showed that arsenic could be found normally in a large number of persons and was dependent upon the character of their food, drink, environment and medication. In a study upon the arsenic content of the blood of human beings at various intervals after the intravenous injection of arsphenamine, they (11) found that immediately after the injection, 60% of the arsphenamine was localized outside of the blood stream and that the blood concentration became gradually lower, but showed a marked increase after one day and then again decreased in arsenic content. In another investigation in regard to the amount of neoarsphenamine in the blood of human beings, they (12, 13) found that 62.1% of the neoarsphenamine was localized outside of the blood stream immediately after the injection, and that the blood arsenic was gradually lowered up to one hour after which it remained almost stationary. In another paper in 1924 Fordyce, Rosen and Myers (14) reported the results of an extensive study on the localization of arsphenamine, neoarsphenamine, silver-arsphenamine and tryparsamide in the viscera of rats after intravenous administration. Their results showed that the amounts of arsenic found in the kidneys, spleen and brain constituted only a relatively small portion of the injected arsenic and that the largest amount of the recovered arsenic at the end of thirty minutes was in the liver and blood. They stated that their results pointed to other sources for storing arsenic, as was indicated by the fact that additional arsenic came back into the blood stream, but they did not carry the investigation further.

Underhill and Dimick (37), in 1928, studied the distribution of neoarsphenamine in the dog. They found that the quantity of arsenic in the liver bore no relationship to the dosage of the arsenical administered and that the kidneys, spleen, thyroid and adrenals showed a high affinity for arsenic. They found, as has Barbat (1), Voegtlin, Smith, Dyer and Thompson (40) and Fordyce, Rosen and Myers (14) that the amount of arsenic in the brain was very small and that neoarsphenamine penetrated the nervous system with great difficulty. Kraft,

Harris, Robinson and Gilliland (21) studied the distribution of neoarsphenamine in the rabbit They concluded that the organ predominantly concerned in the immediate removal of arsenic from the blood stream was the liver, with the kidneys and intestines next in order of efficiency An analysis of the liver, spleen, brain, kidneys, testes and intestine showed a total recovery of 13 2% at the end of one hour, 11 3% at the end of twelve hours, 6 5% at the end of 24 hours, 3 3% at the end of five days, while 1 5% of the injected arsenic was recovered at the end of nine days

Thus the evidence regarding the distribution of the arsphenamines in the body is fragmentary being mainly confined to analyses of those organs which can be removed in toto from the animal body Hence, while a certain percentage of the injected arsenical can be accounted for in this way, the work of practically all previous investigators in this field has accounted for less than one half of the injected drug and in many instances has accounted for not more than 10–20% even at comparatively short time intervals after administration of the drug It appeared, therefore, to be worth while to attempt to account for as much of the injected drug as possible at all time intervals in the case of the whole drug samples, as well as in the case of the separated crystalloid and colloid fractions of arsphenamine and neoarsphenamine

METHODS AND MATERIALS All experiments were carried out on samples of arsphenamine and neoararsphenamine consisting of a single lot number of each drug For the determination of the arsenic content of these drugs the volumetric method of Lehmann (22) as given by the United States Pharmacopoeia (38) and the gravimetric method of Treadwell Hall (36) as modified by Myers and DuMez (27), were used The results which were obtained by these methods are reported elsewhere (30) It was concluded from these analyses that the gravimetric method was the more accurate and consequently it was the method used for the determination of the arsenic content of the crystalloid and colloid fractions of the two drugs after their separation by dialysis

Tissue specimens were digested by the standard procedures for the A O A C Gutzeit method of analysis for arsenic (24) The Gutzeit analyses in the early part of the experimental period were made using the procedure given by Klein (18), who used ferrous ammonium sulfate to bring about the reduction of arsenate to arsenite and to promote a faster evolution of hydrogen This method required that the solutions to be analyzed be heated to 90°C to complete the reduction of the arsenic and subsequently cooled before the test could be made The greater part of the Gutzeit analyses was done using the procedure given in the fourth edition of Methods of Analysis ' by the Association of Official Agricultural Chemists (24) Potassium iodide is used as the reducing agent in this method and offers the advantage that it is not necessary to heat the solution to effect the reduction of arsenate to arsenite A standard graph of the relationship between the number of micrograms of arsenic trioxide and the length of stain was prepared through the use of a standard solution From this graph the amount of arsenic in micrograms found in all tissue analyses was ascertained In those cases where the stain showed an amount of arsenic in excess of ten micrograms the determination was repeated using a smaller aliquot Replicate analyses on known specimens have demonstrated that this method gives results having an accuracy within ±5% for samples not in excess of 500 micrograms of arsenic trioxide (43)

The separation of the arsenical into a crystalloid and colloid fraction was effected by means of dialysis under nitrogen using viscose membranes as developed by Wright et al (45) Rosenthal s test (31) for arsenoxide was used to check for possible oxidation

of the arsphenamine or neoarsphenamine to this substance in the course of the dialysis. All dialysates used for experimental work were negative for arsenoxide by Rosenthal's test.

All the rats employed in the experimental work were either bred in a rat colony maintained jointly by the Department of Pharmacology and the Graduate Medical Research Fund, University of Minnesota, or in a colony maintained by the Department of Physiology and Pharmacology, University of Alberta, Canada, from rats obtained from the Minnesota colony. These rats were of the Wistar strain and were fed a diet which was fully adequate. A total of 308 rats was used involving some 3400 separate tissue analyses in the experiments reported in this investigation.

The solution of neoarsphenamine was prepared by dissolving the drug in water which had been recently boiled and cooled under nitrogen and making the solution up to volume in a glass stoppered volumetric flask. When prepared, it was divided into smaller quantities in 5 or 10 cc. pycnometers, which were completely filled and stoppered. This served to preserve portions of the solution until needed, as the series of injections progressed. Solutions of sodium arsphenamine were made by dissolving the arsphenamine hydrochloride in water previously boiled and cooled under nitrogen, in a 125 cc. Erlenmeyer flask attached to the nitrogen purification chain. The required amount of sodium hydroxide was then added to the flask containing the arsphenamine while still under nitrogen. The solution was maintained under nitrogen and injection samples were taken as needed during the period of injections. Alkalinized arsphenamine solutions are so sensitive to oxidation that formation of detectable amounts of arsenoxide can be prevented only by rigid adherence to this procedure. The separated crystalloid and colloid fractions were similarly maintained under nitrogen and quantities for injection were taken as needed.

The animals were anesthetized lightly with ether and the injections of the arsenical were made intravenously into the saphenous vein. The volume of solution injected seldom exceeded 1 cc.

Blood and tissue samples were taken for analysis at the end of $\frac{1}{2}$, 2, 6, 12, 24, 168 and 336 hours after injection. Blood was collected into 1 cc. of sodium oxalate solution and a 4 cc. sample was used for analysis. The liver, spleen, kidneys, brain, stomach, small intestine and large intestine were removed and the entire specimen used for analysis. The portions of the gastro-intestinal tract were thoroughly washed until absolutely free of contents. For skin and muscle 5 Gm. samples were removed for analysis. For bone the femur and tibia were employed and were taken from the extremity opposite to that in which the injection was made. In the case of blood, skin, muscle and bone, where samples only were employed, the total amount of arsenic in the entire tissue was calculated from the tables of Jackson given in Donaldson's Tables (4) of standards for the rat. The validity of this procedure for determining the total amount of arsenical in sampled tissues was checked by a series of analyses on entire animals and will be reported in the experimental section.

EXPERIMENTAL. (1) *Toxicity of samples of arsphenamine and neoarsphenamine.* In order to demonstrate that the samples of neoarsphenamine and arsphenamine employed in this study possessed physico-chemical and toxicity characteristics essentially similar to those reported by Wright et al. (45) the drugs were subjected to dialysis and the toxicities of the whole drug and separated crystalloid and colloid fractions were determined.

In the determination of the toxicity of the respective drugs, those animals which lived for seven days were classed as surviving for an indefinite period. The L. D. 50 was calculated by the method of Dragstedt and Lang (5), as used previously by Wright et al. (35, 44).

The results shown in table 1 are in agreement with those obtained by Wright

et al. (45) who also found greater differences between the separated crystalloid and colloid fractions of neoarsphenamine than for those of arsphenamine. As a result of the data obtained it was concluded that the samples of neoarsphenamine and arsphenamine obtained for use in this investigation possessed the desired physico-chemical properties and were, therefore, suitable for injection to determine the distribution of the respective whole drugs and separated crystalloid and colloid fractions in the blood and tissues of the rat.

(2) *Determination of the arsenic content of the tissues of normal rats.* Since the tissues of rats normally contain arsenic derived from their diet, it was necessary

TABLE 1

Determination of L.D. 50 of the samples of neoarsphenamine and arsphenamine employed

SAMPLE	NEOARSPHENAMINE L.D. 50	ARSPHENAMINE L.D. 50
	mgm./kgm.	*mgm./kgm.*
Whole drug	370.9	140.0
Crystalloid fraction	425.0	154.0
Colloid fraction	22.7	103.8

Total number of rats used = 102.

TABLE 2

TISSUE	MEAN VALUE
Blood	16.5900 ±0.890 per cc.
Skin	0.4187 ±0.063 per gm.
Muscle	0 4537 ±0.047 per gm.
Bone.	1.5433 ±0.150 per gm.
Liver.	1.1096 ±0.049 per gm.
Kidney.	1.5797 ±0.080 per gm.
Spleen	6.4050 ±0 370 per gm.
Brain.	0.4224 ±0 069 per gm.
Stomach*.	1.5300 per gm.
Intestine*	0.3630 per gm.
Colon*	1.2800 per gm.

* In the case of the stomach, intestine and colon the normal values are based on two determinations only, but agree substantially with the values obtained by Wright (42) on a series of 30 control animals.

to determine the amount of arsenic present in each of the various tissues in which the distribution of the arsenical drugs was to be investigated. This was done on a series of 12 rats taken in pairs at selected intervals during the course of this investigation. The results, expressed as micrograms of arsenic trioxide, are shown in table 2. The mean values for normal arsenic shown in table 2 were applied in the case of each injected rat and all values given are those obtained after deduction of normal arsenic.

(3) *Determination of the distribution and retention of arsenic in the blood and tissues at various time intervals after the intravenous injection of arsphenamine and*

neoarsphenamine in the form of the whole drug and separated crystalloid and colloid fractions. All arsenical preparations were injected at the uniform dosage level of 15 mgm./kgm. This dosage was selected as representing the maximum dose of the most toxic preparation (namely, the colloidal fraction of neoarsphenamine) that could be administered safely (see table 1). Five animals were injected with each preparation for each time interval. Analyses of the gastro-intestinal tract were not included at the beginning of the investigation and were made on only two animals. All figures throughout the discussion represent the mean percentage of the administered dose found in that particular tissue at a given time interval.

(a) *Distribution of arsphenamine.* (i) *Blood.* The mean values for the arsenic content of the blood from groups of five rats killed at various time intervals after the injection of (1) the crystalloid fraction, (2) the colloid fraction, and (3) the whole drug arsphenamine are shown in figure 1. It may be observed that the amount of the colloid fraction of arsphenamine present in the blood stream was appreciably greater than was the case with the crystalloid fraction at all time intervals studied. At both one-half and two hours after the injection of the colloid fraction more than 31% of the injected drug was still circulating in the blood stream, while in the case of the crystalloid fraction only 19.25% was present in the blood stream even 30 minutes after injection, and this had fallen to 8.75% by the end of two hours. The much greater rapidity of penetration of the crystalloid fraction of arsphenamine into the tissues as compared with the colloid fraction is very apparent.

In the case of the colloid fraction, it may further be noted that there was a secondary rise in the arsenic content of the blood occurring after 24 hours and increasing in intensity even to the end of 14 days, when an average of 24.98% of the injected dose was still circulating in the blood stream. No such secondary rise of any extent was evident in the case of the crystalloid fraction, less than 8% of the injected arsenic being present at all time periods later than 6 hours.

The concentration of the drug found in the blood at any one time is necessarily a reflection of the resultant of two antagonistic influences, namely (1) the affinity of the tissues for the drug, and (2) the rate of excretion of the drug.

The prolonged secondary rise in the blood arsenic level in the case of the colloidal fraction appears to be due to the great length of time that this substance is retained in the tissues, as may be seen by reference to figures 1 and 3B. Following the injection of the crystalloid fraction, tissue retention of the drug is negligible after 12 hours.

Following the injection of the whole drug, it may be observed that at the one-half and two hour time intervals, the blood concentrations fell between the values obtained for the crystalloid and colloid fractions respectively, as might be expected from the fact that the whole drug is a mixture of both fractions. The occurrence of a secondary rise at twelve hours is also to be noted. For the remaining time intervals the blood concentrations appear as negative values. This, of course, is not because the blood did not contain any arsenic at all, but because the blood at these time intervals contained less than the mean normal amount of arsenic previously determined as being present in the blood of uninjected control

animals   This appears to be due to a shift in the distribution of the normal arsenic, for the tissue retention graph (fig 3B) shows that there is still an excess of arsenic over the normal arsenic present in the tissues at all time intervals   That this finding is not due to experimental error is indicated by the facts that (1) the total recovery of arsenic (including the blood) for any time interval was never less than the total normal arsenic, and (2) a displacement in the distribution of normal arsenic has also been observed in the investigation of the retention of mapharsen in the tissues of the rat (43)

(II) *Skin*   With regard to the skin it may be noted (fig 1) that the penetration of the colloid fraction into this tissue was greater than that of either the crystalloid fraction or the whole drug   The initial high level of 3 66% at the end of ½ hour was maintained to the end of 2 hours, after which there was a gradual fall to 2 58% at the end of 12 hours, while a marked resurgence of arsenic into the skin took place at the 24 hour time interval, when 4 05% was found to be present The skin appears to have a decided affinity for the colloid portion of arsphenamine since 1 50% was still present at the end of 14 days   Following the injection of the crystalloid fraction there was a rapid penetration of the drug into the skin, 3 42% being found in this tissue ½ hour after injection which is almost as high a percentage of penetration as was found initially for the colloid fraction   The amount of the crystalloid fraction present in the skin begins to fall rapidly after the ½ hour time interval and with the exception of a slight rise at the end of 24 hours, this fall continues until at the end of 14 days the amount of arsenic in the skin had fallen to less than the amount previously determined as being normally present   The whole drug occupies a position midway between that of the crystalloid and colloid fraction as the retention was greater than that of the crystalloid fraction but less than that of the colloid fraction, and at the end of 14 days the skin still contained more than the normal amount of arsenic

(III) *Muscle*   The rapid initial penetration of the crystalloid fraction into this tissue is particularly noticeable 7 89% of the injected drug being found in the muscle ½ hour after injection   The arsenic does not remain in the muscle tissue for any appreciable length of time however, having fallen to 5 30% at the end of 2 hours, to 1 54% after 6 hours, and to 0 10% after 12 hours   At all subsequent time intervals the mean arsenic recoveries were all less than the amount of arsenic normally present in the muscle tissue of the control rats indicating that in leaving the muscle tissue the injected arsenic had mobilized and carried along with it a portion of the inorganic arsenic derived from the diet and normally present in the muscle tissue of the rat   It may perhaps be emphasized that although the mean recoveries at the 24, 168 and 336 hour time intervals are all shown as negative values (fig 1) and indeed were less than the normal arsenic values in 11 of the 15 analyses involved the amount of arsenic actually recovered was without exception greater than the amount present in the reagents   For example, in the five animals investigated 336 hours after injection the mean arsenic recovery from the muscle tissue was equivalent to 18 76 micrograms of arsenic trioxide, of which 0 46 micrograms was derived from the reagents leaving a net recovery of 18 30 micrograms   The mean normal arsenic content of the muscle
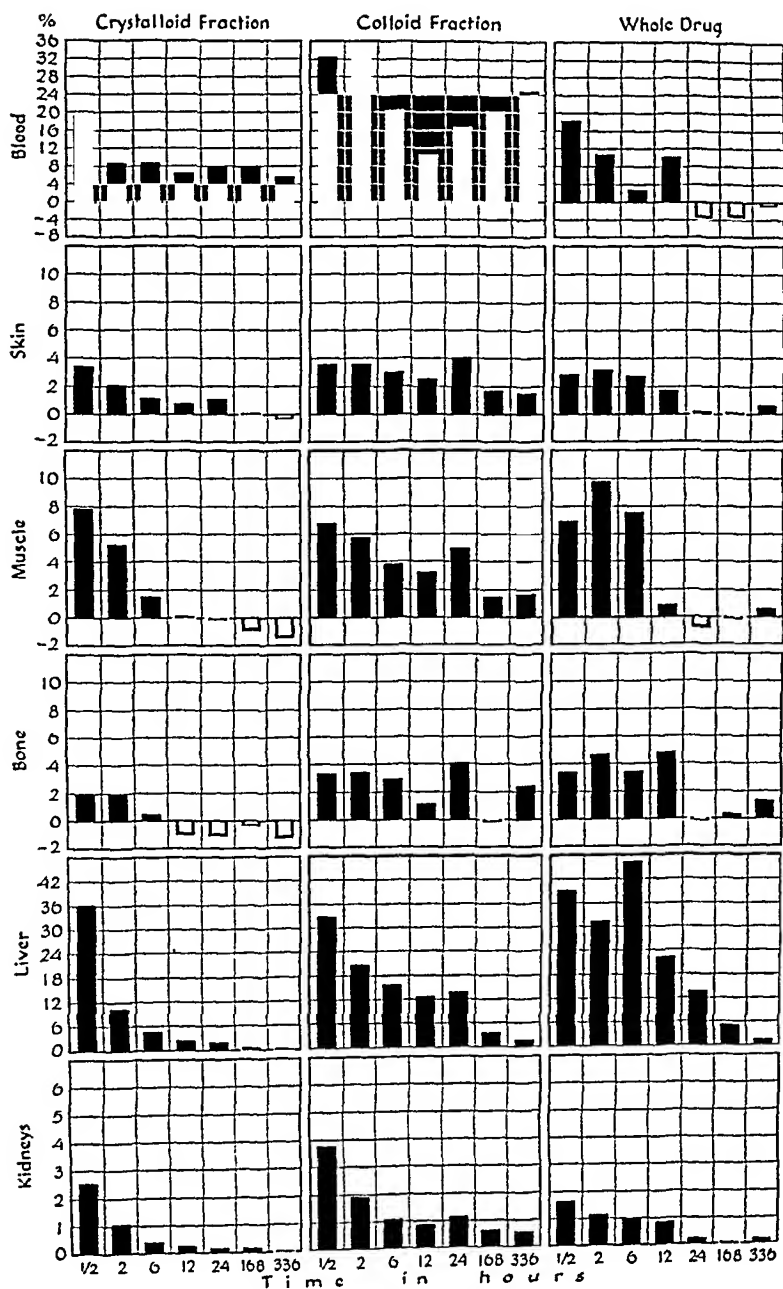
Fig. 1
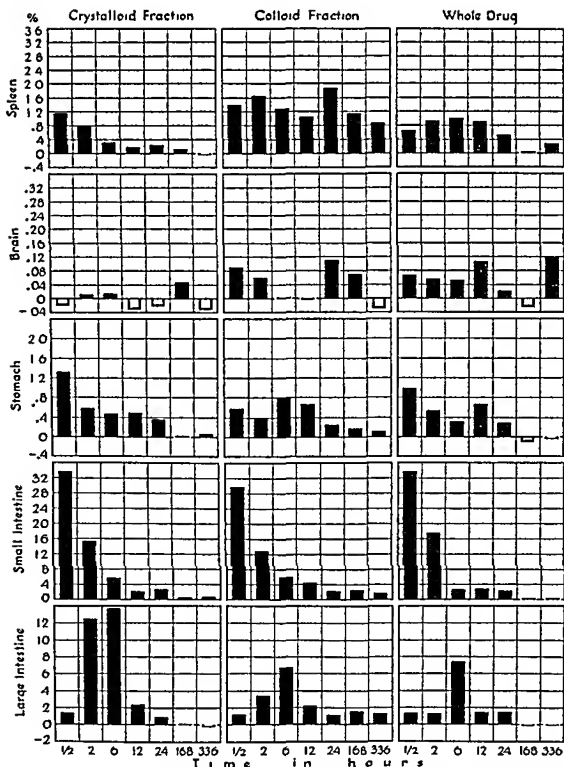
FIG 1. Comparative retention of the crystalloid fraction, colloid fraction and whole drug of arsphenamine following the intravenous injection of 15 mg /kg in the rat

tissue of uninjected control rats of equivalent weight from the same colony was equivalent to 33.93 micrograms of arsenic trioxide, indicating that approximately 54% of the normal dietary arsenic had been "washed out" of the muscle tissue along with the injected arsenic.

The greater affinity of the colloid fraction for the muscle tissue is very evident

from its retention in a higher concentration than that of the crystalloid fraction at all time intervals longer than one-half hour. A moderate secondary rise occurred after 24 hours. In the case of the whole drug the affinity for the muscle was even greater than that of the colloid fraction at the short time intervals (up to 6 hours) but no prolonged retention was found, the behaviour closely paralleling that of the crystalloid fraction.

(iv) *Bone.* The crystalloid fraction of arsphenamine displayed little affinity for bone, approximately 2% of the administered drug being present after $\frac{1}{2}$ and 2 hours, 0.5% after 6 hours, while a negative arsenic balance was found at all later time intervals. The colloid fraction on the other hand was readily taken up by bone, being present in 170, 175, and 630% of the corresponding concentrations of the crystalloid fraction after $\frac{1}{2}$, 2 and 6 hours. A secondary rise occurred after 24 hours and an amount of arsenic equivalent to 2.5% of the administered drug was still present after 14 days. The behaviour of the whole drug closely resembled that of the crystalloid fraction.

(v) *Liver.* The crystalloid fraction showed a high initial ability to be taken up by liver tissue, but it is also comparatively rapidly eliminated from this organ. At the $\frac{1}{2}$ hour time interval 36.05% of the injected arsenic was present in the liver, but this had fallen to 10.35% after 2 hours and to 2.38% after 12 hours, the decline continuing to 1.95% after 24 hours, 0.44% after 7 days and 0.13% after 14 days.

The contrast in behaviour of the colloid fraction is particularly striking (see fig. 1). The initial affinity of the liver for the colloid fraction is not quite as great (approximately 90%) as for the crystalloid fraction, presumably chiefly because of the much greater concentration of the colloid fraction in the blood at this time. The amount of arsenic found at all subsequent time intervals was much greater, however, being 200% of the concentration of crystalloid after 2 hours, 330% after 6 hours, 535% after 12 hours, 700% after 24 hours, 825% after 7 days, and 1415% after 14 days.

The behaviour of the whole drug with respect to the liver not only closely parallels that of the colloid fraction, but the presence of the mixture of both colloid and crystalloid material, such as occurs in the whole drug, appears to result in a very definite exaggeration of the retention of arsenic by the liver. In view of the rapid elimination of the crystalloid fraction from the liver these findings appear to constitute an additional confirmation of our previous demonstration (45) of the desirability of employing in clinical practice samples of the arsphenamines consisting chiefly (if not entirely) of crystalloid material.

(vi) *Kidneys.* The kidneys, while showing the presence of considerable arsenic, account for only a small per cent of the retained arsenic, as would be expected because of their small size in their relation to the tissues of the animal as a whole. The colloid fraction is retained to the greatest extent, 3.77% at the end of $\frac{1}{2}$ hour and remains consistently higher than either the whole drug or crystalloid fraction throughout all the time intervals considered (fig. 1). Following the high initial retention of the colloid fraction, the percentage falls progressively to 1.91, 1.09 and 0.83% at 2, 6 and 12 hours respectively, with a subsequent rise to

1 13 at the 24 hour interval, following which the level of retention drops to 0 63% at the end of 7 days, and to 0 58% after 14 days The crystalloid fraction agains shows a rapid penetration (2 57% at the end of ½ hour), and then a rapid lowering of the retained arsenic to 0 23% at the end of 12 hours, as compared with 0 82% for the whole drug and 0 83% for the colloid fraction Traces of the crystalloid fraction are still present at the end of 14 days Retention by the kidneys of the whole drug occupies a position intermediate to that of the crystalloid and colloid fractions

(vii) *Spleen* There is again a striking difference between the extent to which the crystalloid and colloid fractions of arsphenamine are retained by the spleen The crystalloid fraction reaches its maximum concentration (1 16%) within the first ½ hour, falling progressively to 0 18% after 12 hours, with little change thereafter to 7 days After 14 days three of the five determinations were negative, as was also the mean for the group

The colloid fraction, on the other hand, not only showed a higher initial affinity for the spleen but the substance remained in the spleen in high concentration throughout the 14 day period Expressed as a percentage of the corresponding crystalloid fraction concentrations, the colloid fraction was present in a concentration of 120% at ½ hour, 210% at 2 hours, 400% at 6 hours, 580% at 12 hours, 880% at 24 hours, and 1000% after 7 days Since the crystalloid fraction value was negative after 14 days no comparison with the corresponding colloid fraction can be made, but it is of interest to note that the concentration of the colloid fraction in the spleen at the end of 14 days exceeded the concentration of the crystalloid fraction at all time intervals except ½ hour after injection

The retention of the whole drug by the spleen was intermediate, with a pronounced tendency to follow the pattern of the colloid fraction

(viii) *Brain* The general lack of affinity of the brain tissue for arsphenamine in any form is quite evident The quantities of arsenic recovered from the brain after injection of the crystalloid fraction at no time exceeded an average of more than 0 5 micrograms of arsenic trioxide and may be considered within the limits of error of the method Detectable amounts of arsenic were found at some time intervals after the injection of the colloid fraction or whole drug, but at no time exceeded 0 12% of the administered drug

(ix) *Gastro intestinal tract* The gastro intestinal tract was removed and the stomach, small and large intestines, were thoroughly washed with distilled water until entirely freed of contents The amounts of arsenic reported here were all recovered from the tissue of the gastro intestinal tract itself The contents of the gastro intestinal tract were set aside for subsequent analysis and will be included in a future report

Arsenic was recovered from the stomach tissue at all time intervals following injection of the crystalloid fraction It is not apparent whether the presence of arsenic in the stomach tissue is due to regurgitation of contents from the intestine or to actual excretion into the stomach, and a thorough examination of the role of the gastro intestinal tract in the absorption and excretion of the arsphenamines and mapharsen is now in progress The greatest amount of arsenic recovered

from the stomach tissue, representing 1.32% of the injected drug, was found ½ hour after injection; quantities in the approximate neighborhood of 0.5% were found at subsequent time intervals up to 24 hours, with only traces present after 7 to 14 days.

The amount of arsenic recovered from the tissue of the small intestine was remarkably high, accounting for 33.73% of the administered crystalloid fraction at the end of ½ hour. This fell rather rapidly, however, to 15.29% after 2 hours, to 5.63% after 6 hours, and to 2.01 and 2.63% after 12 and 24 hours, with traces found after 7 and 14 days. Only small amounts (1.36%) were recovered from the tissue of the colon ½ hour after injection, but 12.40 and 13.69% were recovered after 2 and 6 hours. Again it is not clear whether the presence of these large amounts of arsenic in the tissue results chiefly from direct excretion through the gastro-intestinal and colonic mucosa or from excretion of arsenic into the lumen of the gastro-intestinal tract through the bile with extensive reabsorption through the intestinal and colonic mucosa, or whether both processes take place simultaneously in the intact animal. This question is now under active investigation.

No marked difference in behaviour was found following the injection of either the colloid fraction or the whole drug, except that appreciably smaller amounts were found in the tissue of the colon at the 6 and 12 hour time intervals.

(b) *Distribution of neoarsphenamine.* As was the case with arsphenamine the results presented for neoarsphenamine represent the mean of five determinations for each time interval and each tissue except the gastro-intestinal tract for which only two determinations were made.

(i) *Blood.* By reference to figure 2 it may be noted that the amount of the colloid fraction of neoarsphenamine present in the blood, as with arsphenamine, was at all times considerably greater than was the case with the crystalloid fraction. While the concentration of the crystalloid and colloid fraction in the blood both reach a minimum at the 12 hour time interval, the amount of the colloid fraction present is never less than normal, and at the end of 14 days, 20.18% of the injected colloid fraction was still circulating in the blood stream, as compared with only 5.83% for the crystalloid fraction. Here again, as with the colloid fraction of arsphenamine, the prolonged secondary rise in the blood arsenic level in the case of the colloid fraction of neoarsphenamine appears to be due partly to the great length of time that this substance is retained in the tissues and partly to the direct affinity of the blood for the arsenical, as may be seen by reference to figure 3.

Following the injection of the whole drug the blood concentration fell rapidly from the peak of 22.89% at ½ hour, became negative at the 6th hour and remained negative at all subsequent time intervals. The significance of these negative values has been discussed previously.

(ii) *Skin.* The crystalloid fraction showed an initial penetration into the skin to the extent of 6.61%, which was greater than that obtained for either the whole drug or colloid fraction. The level of retention of the crystalloid fraction by the skin fell, however, to 2.92% at the end of 24 hours, and at the end of 14 days the amount remaining in the skin was only 0.17%, as compared with 1.52% for the whole drug and 2.89% for the colloid fraction. This was apparently due to the

more rapid elimination of the crystalloid fraction   The immediate affinity of the skin for the colloid fraction was not as great as for the crystalloid fraction, however, when once attached, it was held more firmly so that the amount retained at the longer time intervals was considerably greater

Following the injection of the whole drug the penetration of the drug into the skin was not as rapid as with the crystalloid fraction, but ultimately became much greater, reaching a peak of 11 14% after 6 hours, and thereafter was retained in the tissue to a greater extent than either the crystalloid or colloid fraction

(III) *Muscle*   By reference to figure 2 it may be seen that the crystalloid fraction of neoarsphenamine penetrated readily into the skeletal muscle tissue, attained its maximum of 7 95% within ½ hour, but showed progressive decrease to 4 72% after 2 hours, 3 35% after 6 hours and 2 18% after 12 hours with a slight secondary rise to 2 68% after 24 hours   After 7 days only 0 41% was present and after 14 days 0 11%   That is, the crystalloid fraction penetrates readily into the muscle tissue after injection, reaching its maximum within one half hour, but is also readily given up again by this tissue

The much greater affinity for and much more prolonged retention of the colloidal fraction in the muscle tissue is in striking contrast to this   Although not reaching quite as high a level in the first ½ hour (95%), after 2 hours the amount of colloid material was 195% of that of the crystalloid fraction at the corresponding time interval, and remained at a higher level at all subsequent time intervals, being 225% after 6 hours, 150% after 12 hours, 130% after 24 hours, 545% after 7 days and 4820% after 14 days

The behaviour of the whole drug after administration paralleled that of the colloid fraction very closely, the values for retention being even somewhat higher than for the colloid fraction at six of the seven time intervals studied

(IV) *Bone*   The retention of the crystalloid fraction by bone follows the pattern characteristic of the crystalloid fraction, namely, a high initial penetration of 9 95% of the injected drug into this tissue at the ½ hour time interval, followed by a sudden decrease to 4 50% at the 2 hour time interval, after which the amount retained is gradually reduced until at the end of 14 days an amount (−0 04%) slightly less than the amount of arsenic normally present was recovered

On the other hand the uptake of the colloid fraction by the bone is most marked   Thus at the end of ½ hour, 8 53% of the injected colloid fraction was localized in the bone, and this uptake was further increased to a maximum of 11 87% at the 2 hour time interval   These high levels were maintained, there being 8 44% present at the end of 12 hours, and at the 24 hour interval the retention had again risen to 10 88%   After 7 days 6 21% was still retained, and even at the end of 14 days 4 93% of the injected colloid fraction was still present in the bone   The bone, therefore, exhibits a very decided affinity for the colloid portion of neoarsphenamine, the retention in terms of the crystalloid fraction at corresponding time intervals being 2 64 times after 2 hours, 4 16 times after 6 hours, 3 08 times after 12 hours, 5 67 times after 24 hours, 4 40 times after 7 days and (based on the mean of the 3 positive determinations for the crystalloid fraction) 20 00 times at the end of 14 days

The retention of the whole drug was intermediate, following the pattern of the
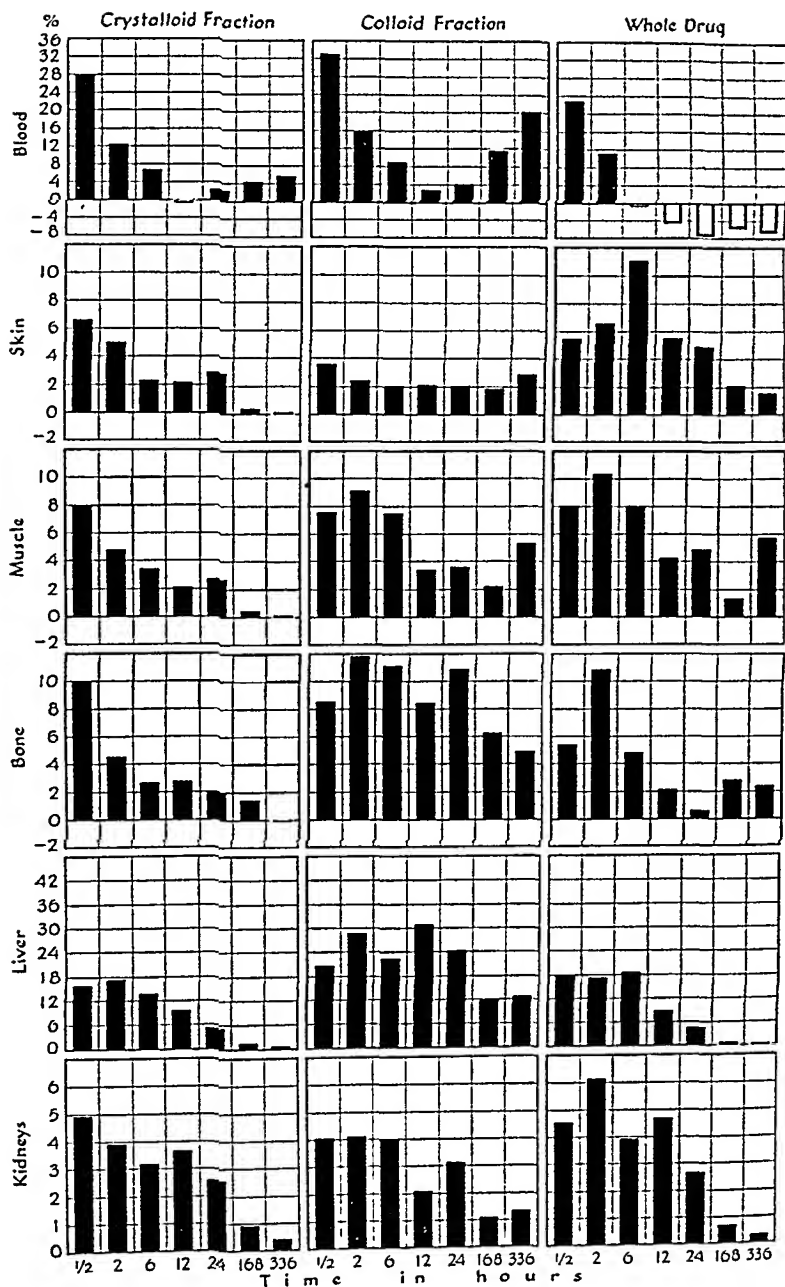
FIG. 2
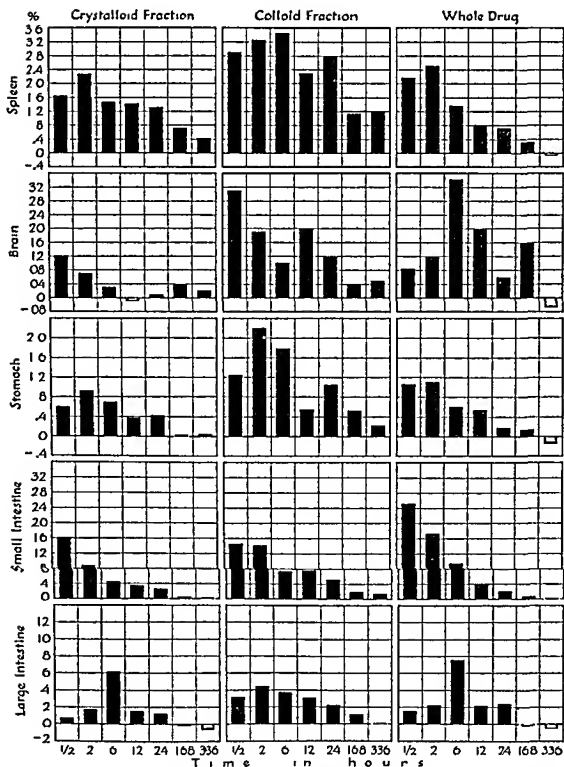
Fig 2 Comparative retention of the crystalloid fraction colloid fraction and whole drug of neoarsphenamine following the intravenous injection of 15 mg/kg in the rat

colloidal fraction at the shorter time intervals, but with a subsequent elimination more nearly corresponding to that of the crystalloid fraction

(v) *Liver* Following the injection of the crystalloid fraction of neoarsphenamine the liver concentration attained a mean level of 15 90% after ½ hour, rose to 17 35% after 2 hours, and then fell progressively through 13 68% after 6 hours,
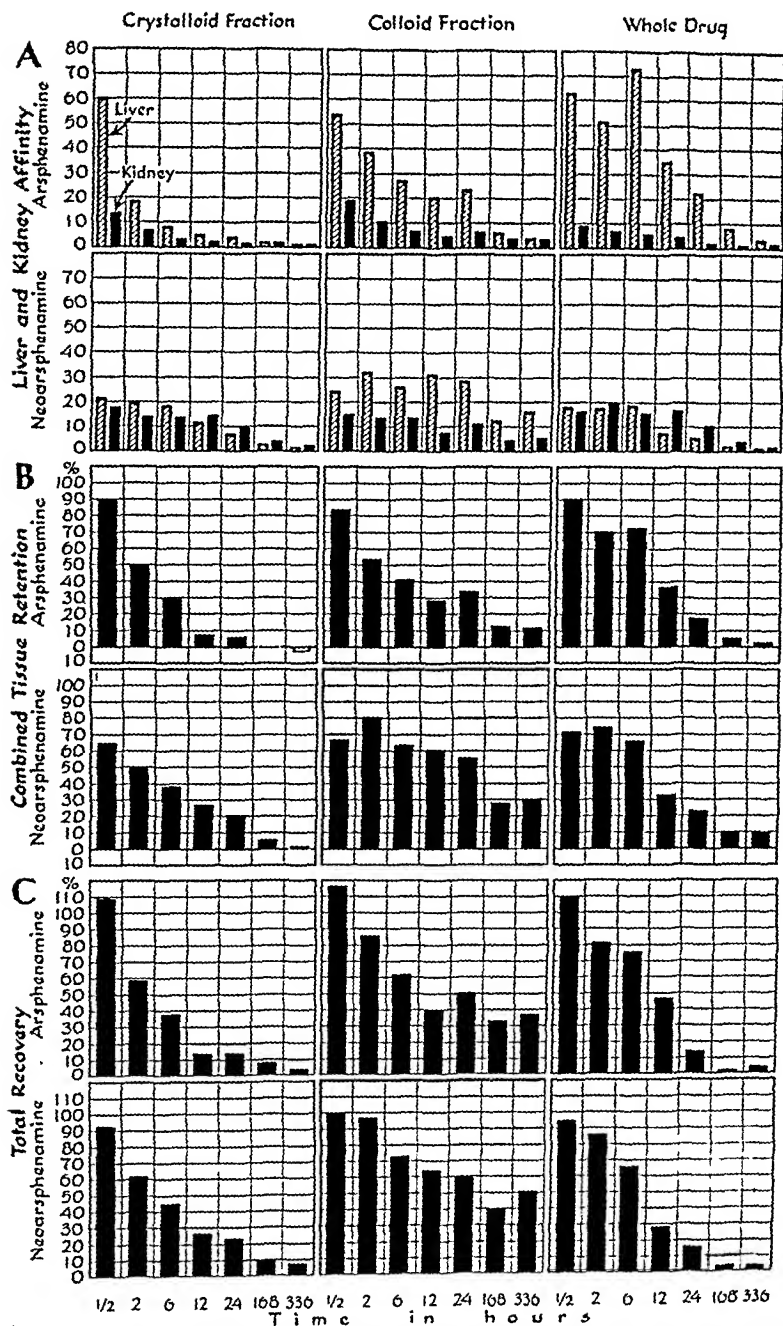
FIG. 3. A. Comparative liver and kidney affinity for arsphenamine and neoarsphenamine. B. Comparative retention of arsphenamine and neoarsphenamine by all tissues (except blood). C. Total recovery of arsphenamine and neoarsphenamine at various time periods.

9 44% after 12 hours and 5 17% after 24 hours   After 7 days 1 37% was found, with 0 63% still remaining after 14 days   The amounts present in the liver after 24 hours were, however, comparatively small

The behaviour of the colloid fraction, on the other hand, was in decided contrast to this, the amounts found being 20 68, 28 78, 22 31, 30 98, and 24 14% after ½, 2, 6, 12 and 24 hours, with 11 94% still present in the liver after 7 days, and 12 70 after 14 days   In a single experiment made with the colloidal fraction 3 37% of the injected drug was found to be still present in the liver after 30 days In terms of the corresponding amount of the crystalloid fraction, the amount of the colloid fraction present was 1 30 times greater after ½ hour, 1 66 times after 2 hours, 1 63 times after 6 hours, 3 28 times after 12 hours, 4 67 times after 24 hours, 8 72 times after 7 days and 20 16 times after 14 days

The amounts of whole drug found were intermediate to those of the two fractions at all time intervals

(vi) *Kidneys*   By reference to figure 2 it may be seen that there is comparatively little differentiation between the crystalloid or colloid fraction with regard to the kidneys, the values at corresponding time intervals being generally within ±20% of each other   In both cases the retention in the kidney was comparatively prolonged, appreciable amounts being still present after 14 days   The retention of the whole drug was somewhat greater than for either of the separated fractions

(vii) *Spleen*   The colloid fraction was retained to a greater extent by the spleen than was the case for either the whole drug or crystalloid fraction   The maximum per cent of the colloid fraction, amounting to 3 46% was found in the spleen at the end of 6 hours, while for both the whole drug and crystalloid fraction, the maximum per cent was found at the end of the 2 hour time interval

(viii) *Brain*   The amount of crystalloid neoarsphenamine present in the brain was maximal with a concentration of only 0 12% after ½ hour, and the amounts recovered after 2 hours were within the limits of error of the method The colloid fraction was present, however, in definitely higher concentration for practically all time intervals   The whole drug behaved somewhat differently from either fraction, reaching a maximum concentration after 6 hours as compared with ½ hour for both fractions

(ix) *Gastro intestinal tract*   Definitely detectable amounts of the crystalloid fraction were recovered from the stomach at all time intervals during the first 24 hours   The amount of crystalloid drug found in the tissue of the small intestine ½ hour after injection was very high, being 16 15% of the total amount of administered drug   As mentioned previously under arsphenamine, the significance of the presence of such large amounts of drug in the tissue (not in the lumen) of the gastro intestinal tract is under active investigation in this laboratory   The amount decreased steadily at later time intervals until only traces were recovered after 24 hours   Only small quantities of arsenic were recovered from the tissue of the colon after ½ and 2 hours, but more than 6% of the injected drug was present after 6 hours   None was found present after 24 hours

The colloid fraction was found to be present in a concentration greater than that of the crystalloid fraction in all three portions of the gastrointestinal tract

Typical of the behaviour of the colloid fraction is the fact that it was found to persist in a higher concentration for much longer time intervals. Thus, expressed as a percentage of the corresponding crystalloid concentration, the colloid fractions were found in the following percentages at the respective time intervals:

	TIME (HOURS)						
	$\frac{1}{2}$	2	6	12	24	168	336
Stomach	210	240	260	150	250	1770	575
Small intestine..	90	160	165	225	190	615	1110
Large intestine .. .	470	260	60	210	190	∞	∞

The whole drug behaved essentially in a manner intermediate to the two fractions, particularly in the stomach and colon. In the small intestine the peak of 25.11% was attained in $\frac{1}{2}$ hour, with 17.20% present after 2 hours and 9.42% after 6 hours. At later time intervals the amounts found approximated those for the fractions.

(c) *Tissue retention and total recovery.* Having available the quantitative recoveries from eleven separate tissues for five animals (except as noted) for each time interval, it appeared of interest to determine how closely the summation of the amounts recovered from all of these tissues would account for all of the administered arsenic, particularly at the shorter time intervals. The results of these summations are shown in figure 3C. Also, because of the very evident shifts of arsenic distribution between blood and tissues at various time intervals (see figs. 1 and 2, blood) it appeared desirable to present a summation of the amount of arsenic recovered from all tissues exclusive of the amount circulating in the blood stream. These values of tissue retention are shown in figure 3B.

Summation of the individual recoveries from the eleven tissues investigated gave a mean recovery at the half-hour time interval of 103.4% for all six samples investigated. That considerable elimination (largely into the gastrointestinal tract) had taken place by the end of two hours is evident since at this time interval the mean total recovery was only 78.1%. The graphs for total recovery from all tissues likewise bring out the striking contrast between the extent of retention of the colloid fraction as compared with the crystalloid fraction and the general tendency of the whole drug to follow the pattern of the colloid rather than that of the crystalloid fraction, a fact pointing to the normal high degree of colloidality of both of the arsphenamines.

As a check on the accuracy of the determination of total recovery by summation of the recoveries from a group of tissues, several of which were determined by calculation from Donaldson's Tables (4) in order to arrive at total tissue arsenic content, a series of five rats was injected with a dosage of 15 mgm./kgm. of neoarsphenamine, sacrificed $\frac{1}{2}$ hour after injection and, after removal of the entire gastro-intestinal tract the animals were digested in toto as single samples. Five uninjected rats were treated in a similar manner to determine normal arsenic under identical conditions. After trial analyses had been made to determine the

most suitable size aliquot, a series of ten Gutzeit analyses was made on each sample, and the arsenic recovery was calculated from the mean of each series These analyses showed a mean arsenic recovery of 63 93% from single sample analyses as compared with a mean recovery of 66 63% obtained by summation of individual tissue analyses exluding the gastrointestinal tract

The graphs (fig 3B) showing the total retention in all tissues except blood show essentially the same findings as for the total recoveries, but with greater contrast between the marked tissue retention of the colloid fraction and the rapid tissue loss of the crystalloid fraction

(d) *Comparative liver and kidney affinity* The comparative affinity of the two drugs and their separated fractions for the liver and kidney was calculated on three bases, namely (1) in terms of the number of times the arsenic content of the organs was increased over the mean value of the normal tissue arsenic, taken as a measure of the normal affinity of these tissues for arsenic, (2) on the basis of the

TABLE 3

Ratios of the comparative affinity of the liver and kidneys for arsphenamine and neoarsphenamine

TIME	GM./CM. BASIS						TOTAL ORGAN BASIS					
	Arsphenamine			Neoarsphenamine			Arsphenamine			Neoarsphenamine		
	Cryst	Coll	W.D	Cryst	Coll	W D	Cryst	Coll	W D	Cryst	Coll	W D
hrs												
½	3 50	2 10	5 24	0 87	1 38	0 70	14 02	8 74	23 45	3 24	5 10	3 95
2	2 07	2 84	5 57	0 80	1 87	0 60	9 60	10 85	27 00	4 45	7 03	2 82
6	2 46	3 12	10 58	0 99	1 47	0 97	12 08	14 40	49 80	4 28	5 68	4 79
12	2 50	3 67	7 14	0 57	3 35	0 44	10 35	15 38	27 05	2 58	14 75	1 88
24	2 78	2 81	13 18	0 49	1 98	0 31	12 19	12 00	62 45	2 00	7 56	1 62
168	0 75	1 54	55 00	0 36	2 30	0 33	2 93	5 77	254 00	1 47	10 75	1 28
336	2 96	0 82	1 89	0 43	2 24	0 10	13 00	3 17	92 30	1 70	9 70	0 60

gm /gm arsenic content of the two organs at corresponding time intervals, and (3) on the basis of the respective total arsenic content of the two organs

On the basis of normal arsenic affinity (fig 3A) it may be seen that the affinity of the liver for arsphenamine generally predominates This is particularly true in the case of the comparatively long retained whole drug and colloid fraction (see fig 3B and C), much less the case with the comparatively rapidly excreted crystalloid fraction except at the earliest time interval With neoarsphenamine the much greater relative affinity of the kidney for the drug is very evident, so that only in the case of the long retained colloid fraction did the liver affinity appreciably exceed that of the kidneys

The ratios for the comparative arsenic content of the two organs (a) on a gm /gm basis and (b) on a total organ basis are shown in table 3 On a gm /gm basis the greater comparative affinity of the liver for arsphenamine is very evident with all three preparations, most of the ratios indicating that the liver retains two to three times as much drug as does the kidney With neoarsphen

amine the ratios bring out even more strikingly than was seen on a normal arsenic content basis the much greater affinity of the kidney for this drug. On a gm./gm. basis the kidney invariably took up more drug than the liver averaging 1.5 times in the case of the crystalloid fraction and 2.0 times as much with the whole drug. Only in the case of the long retained colloid fraction did the liver take up more drug than the kidney.

When compared on a total organ basis the much greater weight of the liver as compared with that of the kidneys makes almost all of the ratios greater than unity, but the same massive affinity of arsphenamine in all forms for the liver and of neoarsphenamine (except with the colloid fraction) for the kidneys again stands out.

These results add confirmation to the findings of Rosenthal (31) who, following the intravenous injection of arsphenamine was able to detect the formation of arsenoxide in the liver but not in the kidneys, and following the injection of neo-

TABLE 4

Neoarsphenamine-arsphenamine ratios for various tissues and time intervals

TISSUE	CRYSTALLOID FRACTION				COLLOID FRACTION				WHOLE DRUG			
	½ hr.	6 hrs.	24 hrs.	14 da.	½ hr.	6 hrs.	24 hrs.	14 da.	½ hr.	6 hrs.	24 hrs.	14 da.
Skin.....	1.93	2.14	2.61	∞	0.98	0.69	0.52	1.85	1.79	4.02	24.35	2.34
Muscle...	1.01	2.18	∞	∞	1.11	1.94	0.73	3.16	1.17	1.05	∞	10.01
Bone.....	5.03	5.96	∞	32.25	2.53	3.85	2.70	1.99	1.60	1.39	∞	1.79
Liver. ...	0.44	2.91	2.65	4.85	0.63	1.43	1.74	6.90	0.46	0.40	0.32	0.11
Kidneys..	1.91	8.18	16.12	37.00	1.08	3.68	2.82	2.26	2.71	4.19	12.14	1.67
Spleen....	1.43	4.59	5.70	∞	2.09	2.68	1.48	1.38	3.29	1.36	1.39	0†
Brain	∞*	3.00	∞	∞	3.44	50.00	1.09	∞	1.14	6.80	3.00	0

* ∞ indicates that the arsphenamine concentration had a negative value.

† 0 indicates that the neoarsphenamine concentration had a negative value.

arsphenamine demonstrated the presence of arsenoxide in the kidneys but was unable to obtain positive tests from the liver.

(e) *Comparative retention of arsphenamine and neoarsphenamine.* Since the same dosage per kgm. was used uniformly for all preparations the results present an interesting opportunity to compare not only the comparative affinity of the various organs and tissues for arsphenamine and neoarsphenamine but also of their respective crystalloid and colloid fractions. The results of such a comparison are shown in table 4. The values are expressed as a ratio of neoarsphenamine:arsphenamine concentrations. Values greater than unity therefore express a greater concentration of neoarsphenamine than of arsphenamine for a given tissue and time interval. Four time intervals, namely ½, 6 and 24 hours and 14 days, were chosen as giving a sufficiently representative cross-section of the distribution throughout the time range. The values for blood and gastro-intestinal tract concentrations have been omitted since these are necessarily antagonistic to and dependent on the other tissue concentrations.

The almost general uniformity of the greater retention of neoarsphenamine over arsphenamine at practically all time intervals is most apparent regardless of whether the respective drugs be compared in the form of the whole drug or of the separated crystalloid or colloid fractions.   The greater retention of neoarsphenamine ranges as high as 40–50 times that of arsphenamine, and for all tissues averages 7.0 times for the crystalloid fraction, 4.0 times for the colloid fraction and 3.6 times for the whole drug.   The only exceptions worthy of note are (a) in the case of the liver, where the whole drug is more strongly retained with arsphenamine than with neoarsphenamine and (b) in the case of the skin, where the retention of arsphenamine predominates with the colloid fraction.

DISCUSSION.   The experiments reported above show that when an arsenical such as arsphenamine or neoarsphenamine is injected intravenously, the physicochemical components of the drug distribute themselves throughout the animal body in a characteristic manner.   The colloid fraction is in general long retained, as it is firmly held by the tissues and slowly returned to the blood stream from which it is eliminated only with difficulty.   Since it has been shown previously (45) that the colloid fraction is of low curative value and high immediate toxicity, it appears that the colloid characteristics of both arsphenamine and neoarsphenamine are essentially undesirable, since this fraction is also largely responsible for contributing to the cumulation of arsenic in the tissues, with the consequent necessity for limitation of the clinical use of these drugs to courses of treatment consisting of 12 to 20 injections.

The crystalloid fraction represents the portion of the arsenical which penetrates the tissues well but which is not long retained and disappears fairly rapidly from the body.   Further, since it has been shown (45) that this fraction possesses a high curative power and a low comparative toxicity, it would appear that from all three standpoints the development of methods for the manufacture of arsphenamine and neoarsphenamine of maximal crystalloid characteristics is highly desirable in the clinical treatment of syphilis.

The fact that in the six samples tested an average total recovery of 103.4% of the injected drug was obtained $\frac{1}{2}$ hour after injection indicates that the organs tested include practically all those containing any appreciable quantity of the drug.   The distribution in the various tissues during the early time intervals shows that the arsenical content of the blood is quickly reduced in the case of the whole drugs and crystalloid fractions the drugs being largely taken up by the tissues, from which they are again comparatively readily returned to the blood stream to be eliminated by way of the kidneys and gastro-intestinal tract.   The relative importance of the role played by the kidneys, liver and especially of the gastro-intestinal tract (where secretion through the gastrointestinal mucosa appears probable in addition to the known elimination through the bile), in the elimination of the arsphenamines cannot be evaluated on the basis of this study. However the analysis of the gastro-intestinal contents (to be reported) shows the presence of appreciable amounts of arsenic.

CONCLUSIONS.   1. The colloid fraction of both arsphenamine and neoarsphenamine is the portion of these drugs which is retained for the longest period of

time. It penetrates into the tissues readily, as is shown by the sharp rise in tissue arsenic in the early time periods, and it also appears to be relatively firmly held by the tissues, since it is reexcreted quite slowly into the blood stream. The arsenic which is returned by the tissues to the blood stream is in turn eliminated from the animal body only with considerable difficulty, since a considerable portion of the injected colloid fraction was found to be still present in the blood stream at the end of thirty days. The greater part of the colloid fraction was found in the blood and liver, but the skin, muscle, bone, and intestinal tract also took up important percentages of the injected colloid fraction. All the tissues examined showed retention of the colloid fraction for a longer period of time than was shown by either the whole drug or crystalloid fraction.

2. The crystalloid fraction showed a high degree of initial penetration of the drug into the tissues with a subsequent rapid lowering of their arsenic concentration. The excretion of the crystalloid fraction from the tissues is much faster than is the case with either the whole drug or colloid fraction.

3. The whole drug occupies an intermediate position, but with a decided tendency to follow more closely the greater and more prolonged retention pattern of the colloid fraction.

4. The distinctive affinity of arsphenamine for the liver and of neoarsphenamine for the kidney has been confirmed and elaborated.

5. When injected into rats in an equivalent dosage neoarsphenamine is retained by practically all tissues (except the blood and gastro-intestinal tract) to a greater extent than arsphenamine regardless of its physico-chemical characteristics.

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SOME PHARMACOLOGICAL ACTIONS OF ADENINE-THIOMETHYL-PENTOSE[1]

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Since nucleic acid derivatives have in recent years come into use for the treatment of certain nutritional and neuritic disturbances, the attempt is being made to find new derivatives which are effective therapeutically and at the same time relatively free from undesirable side effects (1). Clinically, these side effects occur almost immediately after intramuscular or intravenous injection, last only a short time, and are not considered as serious complications. Nevertheless, they are unpleasant to the patient and may include flushed face, hyperpnea, tachycardia, abdominal cramps, feeling of fullness in the head, apprehension, dilated pupils, and slight fall in arterial blood pressure (2).

The pharmacological actions of many nucleic acid derivatives have been reviewed by Drury (3). Euler and co-workers (4) reported on the pharmacology of cozymase, adenosine, adenosine-5'-phosphoric acid (muscle adenylic acid) and adenosine-3'-phosphoric acid (yeast adenylic acid) as tested on blood pressure of rabbits, isolated guinea pig uterus and isolated rabbit intestine. In general, these substances produce cardiac slowing, depression of nodes and conduction of the heart, lowering of blood pressure (largely through direct peripheral vasodilatation), inhibition of intestinal muscle and stimulation of uterine muscle. Members of the series vary somewhat in their relative effects on the different systems and also with the species of animal used for study.

This report concerns the examination of adenine-thiomethylpentose, a lesser known member of the series, isolated from yeast. To better judge the advisability of its use as a therapeutic agent, we have compared this substance with a few other known members of the series with respect to its relative effects on blood pressure and on intestinal and uterine muscle.

Adenine-thiomethylpentose was first isolated from yeast in 1924 by Suzuki, Odake, and Mori (5) and by Levene and co-workers (6). Levene has suggested the following formula:
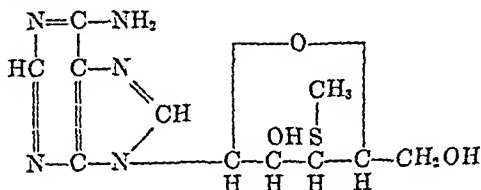
Since the compound so far has been difficult to obtain, investigations on it have been rather limited. It is not known whether it occurs in the animal organism, nor has its biological function been demonstrated so far. It is rather tempting to assume that adenine thiomethylpentose like adenosine may be a degradation product of a nucleotide and as such may be part of some enzyme system.

Some pharmacological properties of adenine thiomethylpentose have been studied recently by Kuhn and Henkel (7). They found that it causes a lowering of the body temperature and basic metabolism of rabbits and cats if given per os in an amount of 150 to 200 mgm per kg.

EXPERIMENTAL PROCEDURES. Adenine thiomethylpentose was prepared from yeast[2] according to the method of Levene (6) with some modifications. It was recrystallized repeatedly from water until the melting point remained constant (210°C.)

Elementary analysis[3] $C_{11}H_{15}O_3N_5S$ (MW 297 1)

Found 43 94% C, 5 23% H, 23 61% N, 11 07% S   Calculated 44 44% C, 5 08% H, 23 55% N, 10 78% S

Fig 1 Blood
(muscle), c 0 25
cozymase f 0 1
moles) Time interval—5 sec

Pure adenosine muscle adenylic acid, yeast adenylic acid and cozymase were prepared according to the methods given in the literature. Solutions of these compounds in distilled water were prepared containing 5 gamma moles per ml. These solutions were used for the following procedures.

Blood pressure of rabbits. The carotid blood pressure of rabbits under dial urethane pentobarbital anesthesia was recorded in the usual manner on a kymograph. Varying amounts of the different solutions were injected intravenously into the exposed femoral vein.

As may be seen from fig 1, adenine thiomethylpentose like other members of the series produced a fall in blood pressure. This depressor effect, however, was considerably less than that produced by any of the other compounds tested. Compared with adenosine a dose of 5 gamma moles of adenine thiomethyl pentose in a rabbit weighing 1 85 kg caused a drop in blood pressure nearly equal to that produced by 0 5 gamma moles of adenosine. It is weaker even than

yeast adenylic acid which has previously been shown to be less active than adenosine (8). Attention should be called to one other factor; that the fall in blood pressure produced by the relatively large doses of adenine-thiomethylpentose is more prolonged than that caused by adenosine and more like that of yeast adenylic acid (9).

Isolated rabbit intestine. The strip of intestine was suspended in a 50 cc. bath of aerated Locke-Ringer solution at constant temperature, and to this bath was added the compound to be tested. Adenine-thiomethylpentose caused an
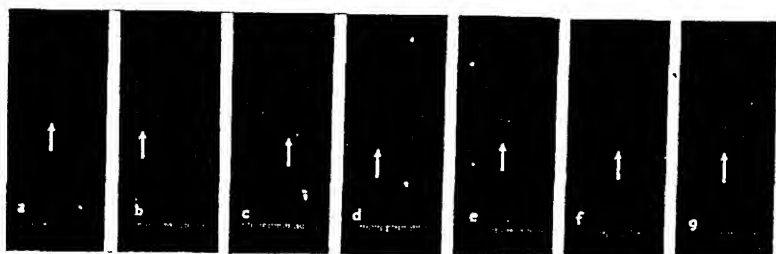
FIG. 2. Rabbit intestine. a, 0.2 cc. adenosine; b, 0.2 cc. adenine-thiomethylpentose; c, 0.1 cc. adenosine; d, 1.0 cc. adenine-thiomethylpentose; e, 0.1 cc. adenylic acid (muscle); f, 0.1 cc. adenylic acid (yeast); g, 0.1 cc. cozymase (1 cc. = 5 gamma moles). Time interval —5 sec.

FIG. 3. Guinea pig uterus. a, 0.1 cc. adenine-thiomethylpentose; b, 0.1 cc. adenosine; c, 0.1 cc. adenine-thiomethylpentose; d, 0.1 cc. adenosine (1 cc. = 5 gamma moles). Uterus I, a, b. Uterus II, c, d. Time interval—5 sec.

inhibition of the intestinal movements as do the other members of the series. Quantitatively, it was considerably weaker in this action; 5 gamma moles produced less relaxation than 0.5 gamma moles of adenosine, indicating a ratio of about 1:15 in activity. Figure 2 shows the response obtained with a representative rabbit intestinal strip.

Isolated guinea pig uterus. Adenine-thiomethylpentose added to the bath caused contraction of the uterine strips similar to the action of the other compounds of this class. The response of different uterine strips varied considerably. As little as 0.05 gamma moles of adenosine was sufficient to initiate contraction of

some strips   In general, the action of adenine-thiomethylpentose on the uterus was approximately as great as that of adenosine.   Attention should be called to the less abrupt response to the adenine-thiomethylpentose as compared to the more marked primary contraction brought about by adenosine.

Enzymatic deamination in vitro.   All substances of this class which exert the pharmacological actions described above have two features in common: the amino group in position 6 of the purine base and a carbohydrate side chain in position 9. As has been found by others, these previously studied substances are subject to enzymatic deamination (3, 10, 11).   Bennet and Drury (8) have stated that it may reasonably be supposed that the substances in question exert their biological activity through the process of deamination.   It seemed of interest to expose adenine-thiomethylpentose to the enzyme deaminase to see whether it would comply with the theory that there is a correlation between pharmacological action and deamination.

TABLE 1

Action of deaminase on adenosine and on adeninethiomethylpentose

SUBSTANCE EXAMINED			INCUBATION TIME	GAMMA MOLES OF NH₃ SPLIT OFF
			minutes	
Adenosine .	.	.	10	2 2
Adenosine	....		30	6.4
Adenosine	.. .	.	60	11 5
Adenosine .	. ... ..	.	120	20.1
Adeninethiomethylpentose			10	0.0
Adeninethiomethylpentose..			30	0 0
Adeninethiomethylpentose		.	60	0 0
Adeninethiomethylpentose		.	120	0 0
Control (no substance)	.	.	120	0 0

Deaminase from calf intestinal mucosa was prepared according to Brady (12). For the deamination experiments, the composition of samples was as follows: 0.04 milli moles of substrate were dissolved in 4 0 ml of 0 06 M citrate-phosphate buffer (pH 7.0).   To this, 1 0 ml. of crude enzyme extract corresponding to 2 mgm of dry preparation was added.   After incubation at 30° C. for various lengths of time the reaction was stopped by adding 1 0 ml of 4 N HCl   The ammonia liberated was distilled off after alkalinization and determined quantitatively.   The results are given in table 1

As can be seen from table 1, adenine-thiomethylpentose is not attacked by adenosine deaminase   This finding, however, does not necessarily exclude the deamination theory of the pharmacological activity.   It is possible that the living organism has an enzyme system which removes the thiomethyl group The adenosine formed could then be responsible for the pharmacological effects observed.

DISCUSSION.   This study of a few effects of adenine-thiomethylpentose shows that it behaves in general in the same manner as adenosine, the adenylic acids,

and cozymase. A fall in blood pressure takes place when a sufficient amount is given intravenously. This effect is maintained considerably longer than that of adenosine. In this respect it is more like yeast adenylic acid (fig. 1, c). In experiments with the isolated intestinal strips of the rabbit, adenine-thiomethylpentose must be given in larger doses than adenosine in order to produce a comparable inhibition, and this inhibitory effect develops more slowly and is more prolonged. The response of the isolated guinea pig uterus is less predictable. Apparently, it varies considerably with the state of the organ. In some instances, the response was slightly greater than that found with adenosine (fig. 3, a), in others it was weaker but in general, about equal. These pharmacological effects suggest that possibly the active substance is a breakdown product and that the release of the active product is delayed and, therefore, prolonged.

SUMMARY

1. Some pharmacological actions of adenine-thiomethylpentose, a naturally occurring nucleoside, were studied.

2. Qualitatively its actions are similar to those of other nucleic acid derivatives of this class, i.e., lowering of the blood pressure in rabbits, relaxation of rabbit intestinal strips and contraction of the isolated guinea pig uterus.

3. Quantitatively, it is much weaker than adenosine in increasing blood pressure and relaxing intestinal muscle and approximately equal to adenosine in *contracting the guinea pig uterus.*

4. Adenosine deaminase does not attack adenine-thiomethylpentose. The significance of this finding in relation to the pharmacological activity is discussed.

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STUDIES ON SHOCK INDUCED BY HEMORRHAGE

IV. The Dephosphorylation of Cocarboxylase in Tissues During Shock and Anoxia[1]

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It has been reported by Govier and Greer (1) and by Govier (2) that the administration of thiamin to dogs in which shock had been induced by hemorrhage prolonged their survival time, and that dogs with a high level of plasma thiamin were more resistant to the onset of shock than were those with a low plasma thiamin   It was also shown that in shock the blood keto acid levels increased (3)

Since dogs which had normal plasma thiamin values were benefitted by thiamin therapy (2), the question arose as to why additional thiamin should be effective One answer to this question would be that the animal s tissue thiamin became incapable of functioning in intracellular metabolism

As is well known, the phosphorylated form of thiamin—diphosphothiamin or cocarboxylase—is the coenzyme essential for the metabolism of pyruvate   The vitamin, thiamin, is apparently absorbed in the free form and becomes phosphorylated to the coenzyme in the cell   In the tissues there is probably an equilibrium between thiamin (and/or its monophosphate) and cocarboxylase as shown in the following reaction

$$\text{Thiamin} + \text{phosphate} \rightleftarrows \text{cocarboxylase}$$
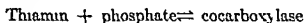
Under normal conditions, in animal tissues most of the thiamin present is in the phosphorylated form but it seemed possible that under abnormal conditions such as in shock, the cocarboxylase might become dephosphorylated, thus shifting the equilibrium to the left   Such an assumption seemed most likely in view of the *in vitro* work of Ochoa (4) who found that in experiments with liver, kidney, muscle and brain under *anaerobic* conditions cocarboxylase was destroyed by an enzyme (probably a phosphatase)   We have repeated and confirmed this work

Since anoxia is known to be one factor in shock, and since cocarboxylase has been shown to be dephosphorylated *in vitro* under anaerobic conditions, it was decided to determine whether cocarboxylase became dephosphorylated in shock induced by hemorrhage, and in dogs made anoxic by reducing the amount of oxygen inspired

Forty-two dogs were used. Shock was induced in nineteen of these, twelve were subjected to anoxic anoxia, and eleven were used as controls. Tables I and III contain results of experiments which are representative of a larger series.

Diet. All dogs used in this series were placed on the same basic diet consisting of cane sugar, casein, cottonseed oil and salt mixture in the proportions recommended by Schaefer, McKibbin and Elvehjem (5) with cellulose flour added for bulk. In addition, all of the animals received the following vitamins twice a week by stomach tube in adequate amounts (5): riboflavin, nicotinic acid, pantothenic acid, pyridoxine and choline, and half of the animals received thiamin. By this method variations in the cocarboxylase content of the tissues were obtained. Ascorbic acid (10 mg.) was also given to each dog along with the other vitamins to protect them from the gangrenous oral lesions already described (6).

Anesthetic. The dogs were anesthetized with pentobarbital sodium as described previously (2).

Production of shock. Shock was produced by the method already described (2) which consisted of arterial bleedings at half hour intervals until the blood pressure remained below 60 mm. Hg for 30 minutes. The amounts of blood removed were as follows: two bleedings of 1% of body weight, two bleedings of 0.5%, and all succeeding bleedings of 0.25%.

Production of anoxia. The dogs in which anoxia was to be produced were connected by a tracheal cannula to a Sanborn metabolimeter filled with a mixture of 20% oxygen in nitrogen. The oxygen content of the inspired gas was gradually reduced to 10% and maintained at this level for one to four hours by means of a Foregger gas machine. If the dogs ceased breathing on this mixture they were given artificial respiration until breathing became normal, and then allowed to breathe air for a few minutes before being returned to the 10% oxygen. Carbon dioxide was removed by means of a soda lime canister.

Tissues analyzed. Analyses for cocarboxylase and total thiamin were done on samples of liver, duodenum and muscle. These tissues were chosen because of their ease of access and because of their mass. According to Leong (7) the total storage of thiamin in the muscle of the rat accounted for about 50% of the total reserves in the body while that in liver amounted to about 35%. If these results be applicable to the dog, changes in the liver and muscle would give a good idea of what happens to the cocarboxylase in the whole animal. In agreement with the results of Goodhart and Sinclair (8) we found no cocarboxylase in blood serum. This can be explained by the finding in this laboratory of an active phosphatase in dog serum which hydrolyzes cocarboxylase (9).

The first tissue samples in all cases were removed after the dogs had been anesthetized for one hour. In the dogs subjected to shock the second samples were taken after the blood pressure had remained below 60 mm. Hg for 30 minutes and the third usually one hour after thiamin administration. In the animals subjected to anoxic anoxia the second samples were taken after the dogs had been respiring a 10% oxygen mixture for one to four hours. In the control animals the second tissues were removed two hours after the removal of the first.

Muscle samples were taken with scissors from the deltoid muscles, liver by means of a cork borer, and duodenum by means of scissors. Hemostasis was obtained by packing the wounds with gauze pledgets in the cases of muscle and liver, and by suturing the duodenum. Muscle and liver were freed from excess blood with filter paper. All tissue samples were frozen immediately in solid carbon dioxide and kept frozen until analyzed. The tissues were then weighed, snipped with scissors and homogenized in distilled water at 0°C. They were then diluted with distilled water so that the resulting suspension contained one gram of tissue in 10 cc. These suspensions were heated at 100°C. for 5 minutes, centrifuged, and the supernatant fluid used for analyses.

Controls. Eleven dogs were used as controls. Samples of muscle only were taken, in order to eliminate any possible shocking effect of visceral manipulation.

Analyses. Total thiamin was determined by the yeast fermentation method of Schultz, Atkin and Frey (10). Cocarboxylase was determined by the Warburg technic using the

split enzyme prepared as described by Green et al (11)   Cocarboxylase is expressed as γ of thiamin per gram of dry tissue in Tables I, II and III   All results of analyses recorded are the averages of duplicate determinations

RESULTS   *Dephosphorylation of cocarboxylase in shock*   Table I shows that in shock induced by hemorrhage a decrease of cocarboxylase occurs in muscle, liver and duodenum   The product formed from cocarboxylase is either thiamin or thiamin monophosphate since in *in vivo* experiments in which determinations of thiamin were done by the Schultz, Atkin and Frey method and in which no diffusion of thiamin occurred, the total thiamin content remained constant even though cocarboxylase decreased   This was also the case in *in vitro* experiments in which cocarboxylase was hydrolyzed anaerobically   The dephosphorylation occurs most frequently in muscle, less frequently in duodenum and in about half the experiments with liver   The number of cases in which dephosphorylation occurs are 12 out of 13, or 92% of the cases for muscle, 9 out of 13, or 69% of the cases with duodenum, and 6 out of 13, or 46% of the cases with liver   The magnitude of the dephosphorylation is variable, there being some tendency for a greater degree of dephosphorylation to occur in dogs which required small amounts of bleeding to go into shock than in those animals which were resistant to shock

In this investigation, however, no attempt has been made to correlate dephosphorylation with other factors, but simply to show that the cocarboxylase content of tissues does change in shock and in anoxic anoxia   Since a great many factors are probably involved, such as the degree of anoxia, the tissue thiamin level of the dog, as well as the ability of various other oxidative enzyme systems to function normally, the problem is a complicated one, and one would expect to obtain large variations among the experiments

When the magnitude of the changes in the muscle of the controls (Table II) is compared with that of the muscle of the shocked animals (Table I) it will be seen that the dephosphorylation of cocarboxylase is much greater in the shocked animals   On statistical analysis, comparing these two groups with reference to muscle samples t = 3 47 when n = 22, thus making the probability of chance occurrence well below 0 01

Resynthesis of cocarboxylase following thiamin therapy   In Table I the percentage increase of cocarboxylase after therapy is calculated on the basis of the cocarboxylase level in shock   Cocarboxylase is resynthesized in from 30 to 60 min following the administration of thiamin, the amount resynthesized frequently exceeding that which was originally present in the animal at the start of the experiment

Dephosphorylation of cocarboxylase in anoxic anoxia   Table III contains representative experiments of this group   It is seen that the changes produced in the cocarboxylase content of tissues of dogs subjected to anoxic anoxia are similar to those found in shocked animals   Dephosphorylation occurred in 92% of cases for muscle (11/12), 67% for liver (8/12) and 82% for duodenum (9/11)   It thus appears that anemic anoxia produces a tissue anaerobiosis similar to that produced by anoxic anoxia and that the anaerobiosis is one factor in both shock and anoxic anoxia which produces the dephosphorylation of cocarboxylase

TABLE I

Dogs subjected to hemorrhage

Cocarboxylase in γ/gram dry weight of muscle, liver and duodenum before and during shock and after thiamin therapy.

DOG	MUSCLE		LIVER		DUODENUM		AMOUNT OF BLEEDING IN PER CENT OF BODY WEIGHT
	Cocar-boxylase	% change	Cocar-boxylase	% change	Cocar-boxylase	% change	
No. 61, 6.25 kg., ♀							
Before shock.................	5.57		10.90		6.88		
After 30 min. shock...........	2.10	−62	5.90	−46	1.21	−83	2.3
After thiamin therapy.........	4.47	+113	10.00	+69	4.74	+147	
No. 63, 11.0 kg., ♀							
Before shock.................	8.26		14.00		6.95		
After 30 min. shock...........	0	−100	9.14	−35	2.40	−66	2.25
After thiamin therapy.........	0	0	6.03	−34	5.90	+146	
No. 77, 11.5 kg., ♀							
Before shock.................	7.62		21.5		12.55		
After 30 min. shock...........	6.06	−14	16.15	−25	10.70	−15	4.45
After thiamin therapy........	10.4	+72	26.7	+65	17.10	+60	
No. 81, 10.1 kg., ♀							
Before shock................. ..	4.24		9.70		4.24		
After 30 min. shock...........	3.62	−15	11.00	+13	4.40	+4	3.25
After thiamin therapy.........	4.26	+18	16.24	+48	4.42	0	
No. 69, 11.0 kg., ♂							
Before shock.................	2.22		5.25		2.67		3.2
After 30 min. shock...........	2.16	−3	5.77	+10	3.40	+27	
After thiamin therapy.........	3.14	+45	8.35	+45	5.66	+66	
No. 45, 14.25 kg., ♀							
Before shock.................	5.52		7.18		2.20		3.2
After 30 min. shock...........	3.14	−43	4.27	−41	1.94	−12	
After thiamin therapy........	5.67	+80	11.40	+167	9.10	+368	
No. 49, 10.0 kg., ♀							
Before shock.................	8.50		12.40		14.40		
After 30 min. shock...........	6.88	−19	14.65	+18	12.20	−15	2.75
After thiamin therapy.........	10.30	+50	21.50	+47	20.30	+66	
No. 51, 15.2 kg., ♂							
Before shock.................	22.10		28.00		13.75		
After 30 min. shock...........	20.30	−8	28.30	+1	23.90	+74	5.45

TABLE I—*Concluded*

DOG	MUSCLE		LIVER		DUODENUM		AMOUNT OF BLEEDING IN PER CENT OF BODY WEIGHT
	Cocar boxylase	% change	Cocar boxylase	% change	Cocar boxylase	% change	
No 59, 8 8 kg , ♀							
Before shock	1 61		4 60		5 02		
After 30 min shock	1 86	+16	11 25	+144	3 97	−21	5 25
After thiamin therapy	5 12	+176	12 45	+11	3 12	−21	
No 67, 10 7 kg , ♂							
Before shock	4 38		11 55		4 29		
After 30 min shock	1 82	−58	10 95	−5	4 06	−5	2 2
After thiamin therapy	2 71	+49	14 65	+34	3 77	−7	
No 71, 8 7 kg , ♀							
Before shock	3 70		8 96		3,62		
After 30 min shock	1 97	−47	8 50	−5	2 84	−22	2 4
No 79, 17 3 kg , ♀							
Before shock	2 78		10 25		4 65		
After 30 min shock	1 88	−32	12 00	+17	3 73	−20	4 2
No 85, 11 2 kg , ♀							
Before shock	2 25		4 94		2 15		
After 30 min shock	1 15	−49	4 95	0	2 37	+10	3 75
After thiamin therapy	3 50	+204	14 35	+190	5 36	+126	

TABLE II
Control dogs

DOG	COCARBOXYLASE IN γ/GRAM OF DRY MUSCLE		
	After 1 hr anesthesia	After 3 hrs anesthesia	% change
110 (1)	4 56	7 44	+63
110 (2)	6 54	9 90	+51
110 (3)	3 67	7 28	+98
110 (4)	6 22	6 06	−3
110 (5)	6 98	8 24	+18
110 (6)	9 35	9 24	−1
111 (1)	5 68	5 16	−9
111 (2)	7 36	6 52	−11
111 (4)	3 29	3 45	+5
111 (5)	4 98	4 32	−13
111 (6)	3 64	4 34	+19

DISCUSSION. Since the time when it was first observed in this laboratory that thiamin can exert a beneficial effect on animals in which shock has been induced by hemorrhage, attempts have been made to discover why this should occur. One reason is that after cocarboxylase has been hydrolyzed in shock it is again resynthesized following thiamin therapy. It would appear that in these conditions a fundamental cause of the dephosphorylation of cocarboxylase is the tissue anaerobiosis produced. It would also seem likely that such a dephosphorylation would also occur in other conditions involving anoxia of the tissue.

The question arises as to why additional thiamin should be required for resynthesis of cocarboxylase. This remains unanswered, but it has been observed that in dogs which went into shock with small amounts of bleeding the plasma

TABLE III

Dogs subjected to anoxia

Cocarboxylase in γ/gram dry weight in muscle, liver and duodenum before and during anoxia.

DOG	MUSCLE		LIVER		DUODENUM	
	Cocar-boxylase	% change	Cocar-boxylase	% change	Cocar-boxylase	% change
No. 47, 11.5 kg., ♀						
Before anoxia	11 20		21 00		16 70	
After 3 hrs. anoxia	8 53	−24	20.60	−2	12 30	−26
No. 73, 9.6 kg., ♂						
Before anoxia	2 97		8 66		3 67	
After 2 hrs. anoxia	1 90	−36	9 82	+13	3 95	+8
No. 75, 11.0 kg., ♂						
Before anoxia	3.29		6.62		5.34	
After 4 hrs. anoxia	1 68	−49	7.20	+9	3 65	−32
No. 57, 10.9 kg., ♂						
Before anoxia	2 42		1 16		5 16	
After 3 hrs. anoxia	2 61	+8	1 14	−2	2 63	−49

thiamin level increased, indicating a diffusion of thiamin from the tissues. Thus, in dogs susceptible to shock the thiamin resulting from dephosphorylation of cocarboxylase is removed from its site of action in the cell. Addition of large amounts of thiamin to the circulating fluid might result in a higher intracellular thiamin level. The mechanism for the resynthesis of cocarboxylase under somewhat anaerobic conditions is unexplained. It is known that phosphorylation of thiamin can occur *in vitro* by the transfer of the labile phosphate groups from adenosine triphosphate to thiamin. Under anaerobic conditions adenosine triphosphate is also dephosphorylated. However, in shock anaerobiosis is not complete and it is possible that sufficient adenosine triphosphate remains intact to initiate the reaction in the presence of excess thiamin, which once started would have a cumulative effect on both the synthesis of adenosine triphosphate

and cocarboxylase   There is also no reason to suppose that breakdown of enzyme systems should be limited to pyruvic oxidase   Voronoy and Lipovetska (12) have shown an increase in dehydroascorbic acid in liver, adrenals, and pituitary in shock, with a concomitant decrease in ascorbic acid, thus showing that this enzyme system is impaired   In a few experiments here, we have noticed a decrease in ascorbic acid similar to that reported by Voronoy and Lipovetska   These investigations are being carried further, as well as investigations of other enzyme systems and the effect of added substrates and adjuvants for phosphorylation

Since pyruvate is an intermediate metabolite for practically all normal cells it can be seen that the specific enzyme required for its metabolism is essential for the normal functioning of those cells   Although a destruction of this enzyme alone might therefore account for many of the widespread and disastrous results occurring in shock other essential enzyme systems are also probably damaged Since, in this system which becomes damaged in shock, the administration of an essential part of the specific coenzyme proves beneficial, it is possible that in other reversibly damaged systems the administration of the appropriate coenzyme would prove equally effective

We wish to point out again that not all of the dogs which we have treated with thiamin recover (2)   This could hardly be expected, since many of the dogs have been bled approximately half of their blood volume, and none of the dogs have been given fluid to replace the blood removed   It stands to reason, however, that if thiamin can produce beneficial results under such adverse conditions, it should be of very much more benefit when combined with measures designed to mechanically improve the circulation, such as the administration of blood substitutes

SUMMARY

1 The cocarboxylase content of tissues of normal dogs, dogs in which shock has been induced by hemorrhage, and in animals in anoxic anoxia has been investigated

2 In shock and in anoxic anoxia a dephosphorylation of cocarboxylase occurs

3 In dogs subjected to hemorrhage thiamin therapy results in a resynthesis of cocarboxylase

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THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON THE TOXICITY OF 2,4-DINITROPHENOL IN MICE[1]

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Several investigators have reported that the toxicity of 2,4-dinitrophenol (DNP) in mammals and birds is decreased at lowered environmental temperatures (1, 2, 3). In particular, Tainter (3) has shown that this is true for the rat and pigeon. Although this observation has been of therapeutic value (4) it has remained qualitative in nature because there has been no statistical evaluation of the actual change of toxicity of dinitrophenol with decrease in environmental temperature. The investigation reported here was undertaken to determine the median lethal dose (LD_{50}) of DNP in mice at 25° and at 6°C., and to show the effect of a high environmental temperature (40°) on the toxicity of the drug.

METHODS. Sodium dinitrophenoxide (*Eastman No. 2077*) was dissolved in buffered Ringer's solution (pH 7.3) and injected subcutaneously. The concentration was varied so that the volume of solution administered remained fairly constant (0.2 to 0.4 ml. per animal). Mice were injected at room temperature, then placed at once in an air thermostat at the experimental temperature. Control animals were placed in the thermostat at the same time. Observations were limited to a two-hour period but animals surviving this period also survived the whole period of the investigation.

Observations were made at three environmental temperatures, 6°, 25° and 40°C., constant to ± 1°C. Graded doses of DNP were administered at each temperature. Each dose was tested on ten adult albino mice. The total number of mice injected was ninety-five (see table 1). Groups of ten uninjected mice served as controls at 6° and at 40°C.

RESULTS AND DISCUSSION. Median lethal doses were calculated by the method of Bliss (5, 6) using the tables of Fisher and Yates (7). The results are presented in table 1, where environmental temperatures, doses of DNP and per cent mortality with each dose are given.

Calculated values of the LD_{50} in mgm. per kgm. of body weight in terms of sodium dinitrophenoxide (with one molecule of water of crystallisation) were 43.41 and 37.55 at 6° and 25°C. respectively. In terms of 2,4-dinitrophenol, the corresponding values were 35.7 and 30.9. The difference between these values is significant (P is less than 0.001).

While sufficient animals were not available to determine the LD_{50} at an environmental temperature of 40°C., it is clear from inspection of table 1 that this dose lies below 10 mgm. sodium dinitrophenoxide (8.22 mgm. DNP) per kgm. body weight. Any such dose is significantly lower than the LD_{50} at 25°C.

The cause of death has not been established in these experiments but some speculation appears warranted by the data presented here and in earlier literature Tainter and Cutting (8) pointed out that there are three possible causes of death from dinitrophenol in acute experiments on homeotherms at room temperature With large intravenous doses (30 or more mgm per kgm) there is direct cardiac depression with rapid fall in arterial pressure With lower doses there was usually progressive and marked increase in body temperature until death ensued from hyperthermia The complete syndrome has been described by Hall and his associates (9) Alternatively, failure of the circulation and of respiration might occur before severe hyperthermia developed In the present study control mice kept at an environmental temperature of 6°C developed progressive hypothermia although they survived the two hour exposure

TABLE 1

ENV TEMP	NO ANIMALS	DOSE	MORTALITY
°C		mgm /kgm *	per cent
6	10	40	30
	10	44	50
	10	48	80
LD₅₀		43 41	
25	10	34	0
	10	36	20
	10	38	60
	10	40	90
LD₅₀		37 55	
40	5	30	100
	5	20	100
	10	10	100
	5	0	0

* Sodium dinitrophenoxide

period At this temperature the body temperature changes in the experimental animals depended on the dosage of dinitrophenol Mice given 20 mgm per kgm became hypothermic but their body temperature remained above that of the controls Metabolic measurements have shown that their oxygen consumption was also above the control level (unpublished work of Turner and Cady, cf also Hall, Crismon and Chamberlin 10 Hall and Chamberlin, 11, and Fuhrman and Field (12, 13) At a dosage level of 30 mgm per kgm with an environmental temperature of 6°C , body temperature fell a little more rapidly in the experimental than in the control animals, indicating that in this dosage and at this temperature the drug interfered somewhat with the cold defense mechanisms Mice receiving 50 mgm per kgm at 6° (ten animals) became hypothermic much more rapidly than the controls and died with body temperatures as low as 21°

or 22°C. There were no survivors at this dosage. However, hypothermia was not the cause of death because untreated mice survived still lower levels of body temperature. The failure of effective cold defense reactions in mice receiving large doses of DNP at low environmental temperature might have been a cause or an effect of circulatory failure similar to that described by Tainter and Cutting (8).

It is suggested that in the mouse at an environmental temperature of 25°C. the LD_{50} dose of DNP produces an augmentation of metabolism which overwhelms the heat loss mechanisms and results in death from hyperthermia. When this sequence of events is prevented by sufficient facilitation of heat loss, a condition which obtains at an environmental temperature of 6°C., a somewhat higher dose of the drug is required to kill by a different action, i.e., depression of the circulation. Further work is planned to clarify this picture.

SUMMARY

1. It is shown that the toxicity of 2,4-dinitrophenol in the adult albino mouse is lowered by decrease and raised by increase of environmental temperature between 6° and 40°C.

2. Calculated values of the LD_{50} for DNP in mgm. per kgm. were 35.7 and 30.9 at 6° and 25° respectively. The difference between these values is significant.

3. The LD_{50} at 40°C. was not determined. However, it is shown that it lies below 8.22 mgm. DNP per kgm. and is significantly lower than the 25°C. value.

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ANESTHESIA

IX THE ANESTHETIC ACTION OF ISOPROPENYL VINYL ETHER[1]

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In a former communication (1) the authors reported studies on the anesthetic properties of cyclopropyl vinyl ether (cyprethylene ether) This compound appeared to be an excellent anesthetic and in a limited number of cases proved meritorious as an anesthetic in man Difficulties encountered in the synthesis of this compound, at present at least, preclude the possibility of its extensive clinical use Continuing our studies of similar ethers as anesthetic agents, our attention was directed to an isomer of cyprethylene ether, namely, isopropenyl vinyl ether The relationship of this compound to cyprethylene ether is apparent from the following formulas
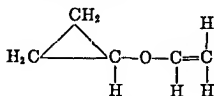
Cyprethylene Ether
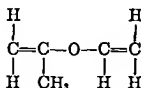
Isopropenyl Vinyl Ether
Propethylene Ether

Propethylene ether involves no great difficulties in its synthesis and has physical properties admirably suited for a volatile anesthetic It is a volatile, colorless liquid with a characteristic ethereal odor, the boiling point is 55°C and the specific gravity 0 786 at 20°C

Anesthesia in the monkey Five large Rhesus monkeys were each anesthetized twice with propethylene ether The technic is described in detail in our former studies (2) The induction period was of short duration, frequently the animals passed into the first plane of surgical anesthesia without appreciable struggle Salivation was not marked and the bronchial tree remained quite free from mucus throughout the anesthesia Surgical anesthesia was uneventful, breathing was stertorous, deep and regular Relaxation of the musculature of the abdomen and extremities was good under surgical anesthesia Only an occasional animal exhibited incoordinated leg movements during anesthesia These occurred near respiratory collapse and were due perhaps to hypoxia Recovery from anesthesias of 30 minutes duration was very prompt, resembling the rapidity of recovery from divinyl oxide anesthesia There was little excitation during the recovery period The quantities of propethylene ether employed were approximately one third the amounts of ethyl ether required for similar anesthetic syndromes

Anesthetic index (dog). The dogs employed were fed a diet of "Purina Chow" one week prior to the experiment and fasted 12 hours immediately before anesthetizing. At least 2 day intervals elapsed between anesthesias in the same animal. The procedure employed was identical with that used in the cyprome ether studies (2). The number of cubic centimeters of the anesthetic agent per kilogram required to produce surgical anesthesia was divided into the volume per kilogram required to produce respiratory arrest. The quotient was designated as the anesthetic index. The results are summarized in table 1.

In our former studies (3) for comparative purposes, experiments were conducted with ethyl ether, cyprome ether, divinyl oxide and chloroform using the same technic. Considering the difference in density, the potency of propethylene ether approaches that of chloroform. The difference between the average induction dose and the average dose producing respiratory arrest for ethyl ether is 1 cc. per kgm.; for propethylene ether, it is 1.26 cc. per kgm.

TABLE 1
Anesthetic index

DOG NO.	SEX	WEIGHT	INDUCTION	RESPIRATORY FAILURE	ANESTHETIC INDEX
		kg.	*cc. /kg.*	*cc./kg.*	
1	F	9.5	0.53	2.11	3.98
2	F	9.8	0.36	1.18	3.28
3	M	7.8	0.38	1.67	4.39
4	M	8.0	0.44	1.50	3.41
5	F	10.0	0.35	1.35	3.86
6	F	10.0	0.35	1.55	4.43
7	M	6.0	0.42	2.25	5.36
8	M	8.1	0.31	1.48	4.78
9	M	7.3	0.34	1.85	5.44
10	F	11.2	0.27	1.47	5.46
Mean..			0.38	1.64	4.32

Blood pressure studies (dog). The effect of propethylene ether on the blood pressure was determined by cannulating the femoral artery under procaine hydrochloride anesthesia and preparing the animal for a blood pressure tracing in the usual manner. The respiratory tracings were made by means of a chest tambour. After obtaining a normal tracing, propethylene ether was administered by the same procedure employed to measure the anesthetic index. In the 2 experiments conducted respiratory failure occurred before cardiac stoppage. The blood pressure was depressed slightly at the plane of surgical anesthesia and deeply at the point of threatened collapse. Another animal was treated in the same manner but the amount of anesthetic was reduced so as to produce the various planes of anesthesia. Portions of this tracing are shown in chart 1.

Electrocardiographic studies (monkey and dog). Three Rhesus monkeys were anesthetized to the surgical plane with propethylene ether for 15, 30 and 70 minutes respectively and cardioscoped during anesthesias.

Five dogs were anesthetized for 30 minutes and permanent tracings of their ECG made. Under propethylene ether anesthesia in the dog and monkey there appeared no significant difference in the form or regularity of the ECG. The rate was generally increased. The R spike, as a rule, was slightly decreased in amplitude. The T wave was flattened frequently in Lead II and occasionally inverted. There were no extraventricular systoles observed. A typical tracing Lead II is shown in chart 2 of the monkey before and under anesthesia.

Effect on the perfused heart (frog). Propethylene ether was dissolved in Howell Ringer's solution and perfused through the frog's heart in situ. Several dilutions
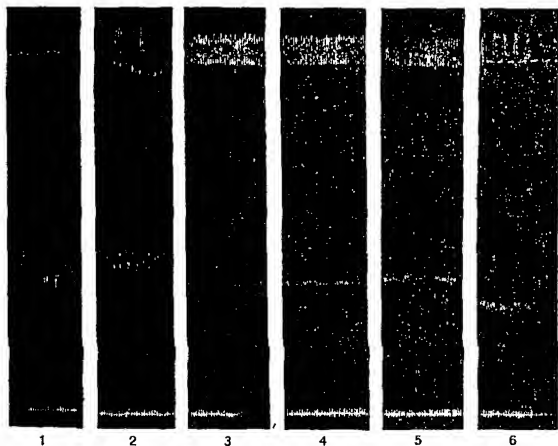
CHART 1 BLOOD PRESSURE OF DOG UNDER PROPETHYLENE ETHER ANESTHESIA
The upper tracing is respiration (1) no anesthesia (2) is the induction period (3) surgical anesthesia after 15 minutes (4) surgical anesthesia after 30 minutes (5) surgical anesthesia after 55 minutes (6) threatened respiratory collapse

were employed to determine the threshold concentration required to produce demonstrable effects within one minute. This concentration in most instances was 0 007 molar or about 60 mg per cent more than double the concentration found in dog's blood under surgical anesthesia. A typical tracing from one of 7 animals is shown in chart 3.

Liver function tests (monkey). Three monkeys were subjected to the bromsulfalein liver function test as set forth in our studies with evprethylene ether (1). The dye excretion period was 30 minutes. Twenty four hours after 30 and 70 minute anesthesias with propethylene ether, the dye excreted was not significantly different from the preanesthetic rate.

Blood chemistry studies (dog). Three dogs were anesthetized to the surgical plane and then given more of the anesthetic until respiratory arrest occurred according to the anesthetic index technic. Prior to anesthesia and 24 hours later blood samples were drawn for analysis. No significant changes in carbon dioxide-combining power or urea nitrogen were observed.

Delayed anesthetic deaths (rat). Fifteen male adult rats were anesthetized with propethylene ether to the surgical plane and maintained in this state for 30 min-
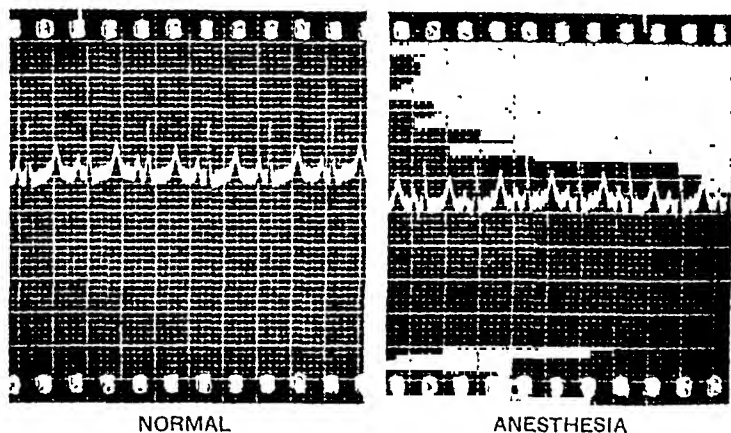
NORMAL   ANESTHESIA

CHART 2. ELECTROCARDIOGRAMS, NORMAL AND UNDER PROPETHYLENE ETHER ANESTHESIA FOR 30 MINUTES (MONKEY LEAD II)

1   2   3   4   5
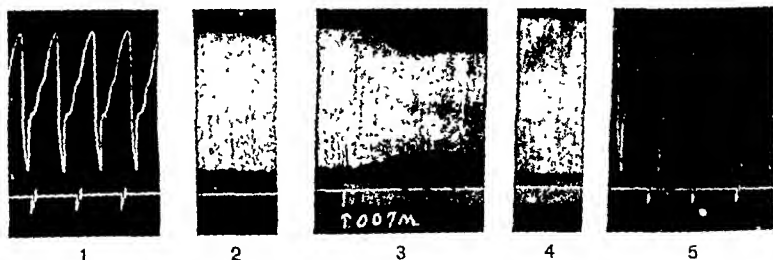
CHART 3. EFFECT OF PROPETHYLENE ETHER ON THE FROG'S HEART

1, Howell-Ringer's solution, fast drum; *2,* same, slow drum; *3,* Seven thousandths molar propythelene ether; *4,* same after 8 minutes, *5,* same, fast drum after 10 minutes.

utes. Four animals were sacrificed at the end of 2 weeks and no significant findings were observed in the liver or kidneys. At the end of 3 weeks none of the remaining animals had died or appeared to be in an unhealthy condition.

Concentration required for anesthesia (mouse). The concentration of propethylene ether required to induce anesthesia was determined by typical partial pressure experiments described previously (2). The results are shown in table 2. With ethyl ether, in our previous studies, 4 per cent partial pressure produced no anesthesias and 5 per cent anesthetized 40 per cent of the animals.

Histological studies of viscera (rat, dog and monkey) Four of the rats used in the delayed anesthetic death studies were sacrificed and their lungs, liver, spleen and kidneys were found to be free from significant changes Five dogs were anesthetized lightly with cyclopropane and liver biopsies performed The anesthetic was changed to propethylene ether and maintained at one hour in 4 cases and 3½ hours in one animal and second biopsies were performed There were no significant pathological findings Two Rhesus monkeys were subjected to the foregoing procedure as performed on the dogs The findings in these 2 animals coincide with those described in the dog anesthesias

Clotting time and hemolysis (monkey and dog) The clotting time of the blood was determined in 2 normal Rhesus monkeys by the capillary tube method The average clotting time was one minute Within the error of the experiment this period was neither diminished nor increased under surgical anesthesia with propethylene ether

Volumes of 10 cc of propethylene ether in varying concentrations in normal salt solution were maintained at 24° and 37°C respectively Concentrations of 21, 42 and 84 mg per cent respectively did not hemolyze red blood cells during the 5 hour period of observation at either temperature A saturated solution of

TABLE 2

Induction concentration in mice

PARTIAL PRESSURE	CC PER LITER	NUMBER OF MICE USED	PER CENT ANESTHETIZED
per cent			
3 0	0 13	20	35
3 5	0 15	20	80
4 0	0 17	27	100

propethylene ether in normal salt solution produced complete hemolysis in 15 minutes at 37° and in 2½ hours at 24°C

Preanesthetic medication (monkey and dog) In monkeys and dogs inducing propethylene ether anesthesia with nitrous oxide or cyclopropane oxygen mixtures was uneventful Preanesthetic medication with pentobarbital sodium or morphine atropine was found to be compatible with propethylene ether anesthesia Ten experiments in all were conducted

Quantitative determination in blood (dog) In acid solution propethylene ether decomposes according to the following equation

$$\underset{\substack{|\\H}}{\overset{\substack{H\\|}}{C}}=\underset{\substack{|\\CH_3}}{\overset{\substack{H\\|}}{C}}-O-\underset{\substack{|\\H}}{\overset{\substack{H\\|}}{C}}=\underset{\substack{|\\H}}{\overset{\substack{H\\|}}{C}} + HOH \underset{\longleftarrow}{\overset{[H^+]}{\longrightarrow}} CH_3CHO + (CH_3)_2CO$$
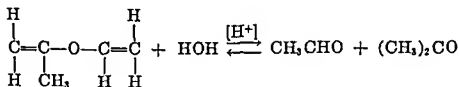
Blood containing the anesthetic agent was hemolyzed and hydrolyzed with acidified water This was distilled into neutral potassium permanganate solution which oxidized the acetic aldehyde but not the acetone The acetone was distilled from the oxidizing agent and determined colorimetrically by the method of

Behre and Benedict (4).   After one hour anesthesia the average concentration (4 experiments) was 24 mg. per cent.   In one experiment at the end of $3\frac{1}{2}$ hours anesthesia, the concentration was 27 mg. per cent, one-half hour, 1 hour, 2 hours, and 3 hours after recovery the blood concentrations were 16, 14, 9 and 7 mg. per cent respectively.

Hydrolysis studies.   Having shown that isopropenyl methyl ether (5) hydrolyzed in blood and that propethylene ether underwent hydrolytic cleavage in acid solution, it was necessary to determine the fate of this agent in the blood.   Propethylene ether failed to decompose by vigorous agitation with phosphate buffer pH 7.4 at 25°C.   In 4 experiments in the dog after one hour's anesthesia, the blood acetone was 3.7 mg. per cent average: the norm was 0.7 mg. per cent average.   In one experiment this was confirmed in the monkey's blood.   In one experiment after 3 hour's anesthesia the blood acetone was 8 mg. per cent and 2 hours after recovery 6 mg. per cent.

Apparently, the molecule of propethylene ether is quite refractory to hydrolysis by the metabolic reactions in the dog, but small quantities are decomposed.

Physical properties.   Solubility in water.   Four cc. of propethylene ether were mechanically agitated with 100 cc. of water for 3 hours at 25°C. in a "Cassia Flask."   The two liquids were allowed to separate for 12 hours and the volume of supernatant ether measured.   The solubility was found to be 0.4 cc. per 100 cc. of water.

Inflammability range.   Mixtures of propethylene ether vapor and oxygen were prepared at 25°C. and at atmospheric pressure in an explosion pipette.   The mixtures were exposed to the spark of an induction coil.   Explosions occurred when the concentration of propethylene ether mixed with oxygen was 2.0 per cent.

Vapor pressure.   The vapor pressure of propethylene ether at 25°C. determined in a nitrometer is 256 mm., that of ethyl ether at the same temperature is 532 mm. (6).

SUMMARY AND CONCLUSIONS

1. Isopropenyl vinyl ether (propethylene ether) isomeric with cyclopropyl vinyl ether is a volatile liquid exhibiting anesthetic properties when administered by inhalation to various species of animals.

2. Propethylene ether exhibits an anesthetic potency which approximates chloroform and an anesthetic index more than twice that of ethyl ether.

3. In the monkey, propethylene ether produces no functional liver damage as shown by the bromsulfalein test.   In these experiments in the rat, dog and monkey anesthesias propethylene ether produced no significant histopathological changes in certain viscera.

4. The monkey's heart showed no significant electrocardiographic changes under anesthesia with propethylene ether.

5. The anesthetic conentration in the blood under surgical anesthesia is approximately one-fifth that of ethyl ether.

6. The blood pressure of the dog is lowered by anesthetic concentrations of propethylene ether.

7. The explosive range of concentrations of propethylene ether and ethyl ether with air appears to be about the same.

8. This first approximation of the pharmacology of propethylene ether, in our opinion, warrants its careful and judicious trial in man by skilled anesthetists.

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ADDENDUM

The experiments having been completed, we deemed that the properties of propethylene ether warranted its trial as an anesthetic in man. On June 30, 1943, at 11.30 A.M. one of us (J. C. K., Jr.) administered propethylene ether to an anesthetist, Constance Black, by the open drop method. The induction period was about 60 seconds. The light anesthesia was continued for 2 minutes. The recovery was rapid and uneventful. The subject stated that the vapors did not irritate the upper respiratory tract.

PHARMACOLOGICAL STUDIES ON *dl-β*-PHENYL-*n*-PROPYLMETHYL-AMINE, A VOLATILE AMINE[1,2]

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In an attempt to find a volatile amine with good vasoconstrictor action, a minimum degree of central stimulatory effect, and relatively low toxicity, a series of compounds which were variations of aryl-ethyl-, aryl-propyl-, or aryl-butylamine structures were synthesized. All the compounds (unless otherwise designated, as the hydrochlorides) were tested in a preliminary manner for pressor effect and toxicity following intravenous administration.

Some of the more interesting compounds studied, along with certain of their properties, are listed in table 1. Included in this list for purposes of comparison are amphetamine and *l*-ephedrine. It will be noted that this series includes primary, secondary, and tertiary amines. Of this group *dl-β*-phenyl-*n*-propylmethylamine appeared to be the most promising with respect to pressor action and toxicity, and it was subjected to a more detailed pharmacological and toxicological study.

CHEMICAL AND PHYSICAL PROPERTIES. *dl-β*-Phenyl-*n*-propylmethylamine is a secondary amine with the following structural formula:
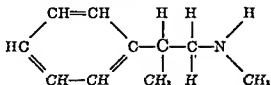
It is a colorless liquid having a characteristic odor, a molecular weight of 149.1, and a boiling point of 211°C. On exposure to air it forms a carbonate from which the amine readily volatilizes. *dl-β*-Phenyl-*n*-propylmethylamine exerts 1 mm. of vapor pressure at 65°C. It is miscible with organic solvents such as ether, benzene, chloroform, alcohol, and acetone; readily soluble in mineral oil and fatty oils, and dissolves in water to give a 1.2% solution at 25°C.

dl-β-Phenyl-*n*-propylmethylamine forms salts readily with mineral acids, a characteristic property of amines. The hydrochloride is a white crystalline solid with a melting point of 144°C. It is very soluble in water and alcohol; slightly soluble in acetone and insoluble in benzene, chloroform and ether. The molecular weight of the hydrochloride is 185.71. It is a stable compound and does not decompose on storage under ordinary conditions of exposure.

RESULTS OF EXPERIMENTAL STUDIES. The pharmacologic and toxic effects of *dl-β*-phenyl-*n*-propylmethylamine have been studied and compared with those of

TABLE 1

COMPOUND NO.	COMPOUND	STRUCTURAL FORMULA	TYPE OF AMINE	MOLECULAR WEIGHT	1 MM. VAPOR PRESSURE AT °C.	L D 50 MGM. KGM I V.— RABBITS	PRESSOR ACTION 1 MGM./KGM. IV.—DOGS
1	dl-β-phenyl-n-propyl-amine	C_6H_5—CH$_2$—CH—CH$_3$ / NH$_2$	Primary	135.1	50	55	+++*
2	dl-β-phenyl-isopropyl-amine (amphetamine)	C_6H_5—CH$_2$—CH(CH$_3$)—NH$_2$	Primary	135.1	50	15–20	+++
3	dl-β-phenyl-β-methyl-isopropylamine	C_6H_5—CH$_2$—C(CH$_3$)(CH$_3$)—NH$_2$	Primary	149.119	38	38	+++
4	dl-β-phenyl-β-ethyliso-propylamine	C_6H_5—CH$_2$—C(C$_2$H$_5$)(CH$_3$)—NH$_2$	Primary	163 13	65	38	++
5	dl-β-phenyl-β-isopro-pylisopropylamine	C_6H_5—CH$_2$—C(CH(CH$_3$)(CH$_3$))(CH$_3$)—NH$_2$	Primary	177 11	60	30	+

	Name	Structure	Type				
6	*dl* β (α thienyl) isopropylamine	α C₄H₃S—C—C—N with H, H, H / CH₃ / H groups	Primary	141 14	42	35	++++
7	*dl* β methyl β (α thienyl) isopropylamine	α C₄H₃S—C—C—N with H, CH₃, CH₃ / H / H groups	Primary	155 16	65	63	+++
8	*dl* β phenyl n butyl amine	C₆H₅—C—C—N with H, C₂H₅, CH₃ / H / H groups	Primary	149 1	65	38	+++
9	β phenyl tertiary butylamine	C₆H₅—C—C—N with H, CH₃, CH₃ / H / H groups	Primary	149 1	45	15-20	++++
10	*dl* β phenyl n propyl methylamine	C₆H₅—C—C—N with H, CH₃, CH₃ / H / H groups	Secondary	149 1	65	65	++++
11	*l* β phenyl β hydroxy isopropylmethyl amine	C₆H₅—C—C—N with H, OH, CH₃ / CH₃ / H groups	Secondary	105 1		65-70	++++

TABLE 1—*Concluded*

COMPOUND NO.	COMPOUND	STRUCTURAL FORMULA	TYPE OF AMINE	MOLECULAR WEIGHT	1 MM. VAPOR PRESSURE AT °C.	LD 50 MGM. KGM. I V.—RABBITS	PRESSOR ACTION 1 MGM/KGM. IV.—DOGS
12	*dl*-(β-phenyl-*n*-butyl) methylamine		Secondary	163.13	65	42	+++
13	*dl*-β-phenylethyl-ethyl-amine		Secondary	149.1	55	78–80	+
14	*dl*-(β-phenyl-*n*-butyl) ethylamine		Secondary	177.14	60	42	– –
15	*dl*-β-phenylethyl-*n*-pro-pylamine		Secondary	163.13	57	53	–
16	*dl*-β-phenylethyl-iso-propylamine		Secondary	163.13	55	70–75	+

| 17 | *dl-β*-phenyl-*n*-butyldi-methylamine | | Tertiary | 177.14 | 65 | 53 | -- |
| 18 | *dl-β*-phenyl-*n*-butyldie-thylamine | | Tertiary | 205.17 | 80 | 40 | -- |

* ++++ is equivalent to the rise in blood pressure elicited by epinephrine hydrochloride in a dose of .0014 to .0018 mgm/kgm. As the number of plus signs decreases there is a corresponding decrease in the pressure.

l-ephedrine, a secondary amine, and *dl-β*-phenylisopropylamine or amphetamine, a primary amine.   The latter compound is also a volatile amine.   Unless otherwise stated all dosages were calculated as mgm. of hydrochloride per kgm. body weight.

Acute toxic effects of *dl-β*-phenyl-*n*-propylmethylamine and effects following prolonged administration of large doses were studied in the rabbit following oral and intravenous administration.   Injections were made into the marginal ear vein at a rate of 50 mgm. per minute, and oral administration was by stomach tube.

The toxic manifestations produced by *dl-β*-phenyl-*n*-propylmethylamine were similar to those elicited by *l*-ephedrine or amphetamine.   Lethal or slightly less than lethal doses whether intravenous or per os, produced characteristic symptoms which included tremors and/or slight convulsions accompanied by an increase in the pulse and respiratory rate.   Recovery was rapid following sublethal doses while larger doses resulted in death due to respiratory and cardiac failure following shortly after the convulsions.

TABLE 2

Acute intravenous toxicity—rabbits

	DOSE (MGM./KGM.)						
	50	55	60	65	70	75	80
No. of animals dead......	0	0	1	5	2	7	10
No. of animals injected	12	12	12	12	12	12	12

The results presented in table 2 indicate that the L.D.$_{50}$ of *dl-β*-phenyl-*n*-propylmethylamine following intravenous administration to rabbits is 65 mgm. By the same procedure the L.D.$_{50}$ of *l*-ephedrine was found to be 65–70 mgm. and that of amphetamine 15–20 mgm.   The acute intravenous toxicity of *l*-ephedrine and *dl-β*-phenyl-*n*-propylmethylamine is, therefore, approximately the same, whereas amphetamine is at least twice as toxic as either of these compounds. Large doses of *dl-β*-phenyl-*n*-propylmethylamine are tolerated orally, 500 mgm. killing only one out of six rabbits.

The effect of prolonged administration of large amounts of *dl-β*-phenyl-*n*-propylmethylamine was also studied in rabbits.   The animals were divided into the following groups: controls, a group receiving the compound in a daily dose of 25 mgm. administered intravenously, and another receiving a daily dose orally of 100 mgm.   All of the dosages in these tests were calculated on the weight of the animals at the beginning of the test.   The compound was administered daily for a period of four weeks and weights were recorded at weekly intervals.   Most of the animals in the various groups showed increases in weight throughout the test period.   Gross and microscopical examination of the tissue revealed no pathological changes which could be attributed to the compound.

Effect on the circulatory system.   *dl-β*-Phenyl-*n*-propylmethylamine has a very definite effect on the cardiovascular system.   Qualitatively this resembles that of

l-ephedrine and amphetamine. In the decerebrated dog, 1 mgm. of the compound produces an increase in blood pressure which is equivalent to that elicited by epinephrine in a dose of 0.00165 mgm. (fig. 1). This increase in blood pressure is quantitatively similar to that produced by either *l*-ephedrine or amphetamine in a dose of 1 mgm. Along with the increase in blood pressure *dl-β*-phenyl-*n*-propylmethylamine produces a decrease in the pulse rate. Since the decrease is prevented by the administration of atropine sulfate it appears to be of central origin.

In the dog anesthetized with morphine sulfate followed by chlorobutanol *dl-β*-phenyl-*n*-propylmethylamine increased the amplitude of the cardiac contractions as determined by direct cardiac tracings. Results of experiments designed to determine the site of action indicate that the compound stimulates the heart both through the stellate ganglia and the peripheral nerve endings.
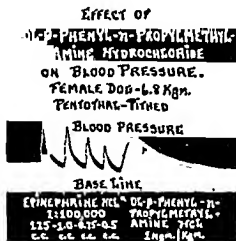
Fig. 1. Blood Pressure in Pithed Dogs

The pressor effect of *dl-β*-phenyl-*n*-propylmethylamine, 1 mgm , compared with that elicited by epinephrine.

In the peripheral circulation *dl-β*-phenyl-*n*-propylmethylamine produces a definite vasoconstriction. This was tested in dogs using Jackson's nasal plethysmograph (fig. 2) and in man by means of the improved Glatzel mirror as described by Lieb and Mulinos (1). In the experiments on man tests were made following the inhalation of vapors of the free amine. Similar studies were carried out with amphetamine and the results of these comparative studies indicate that the two compounds produce approximately the same degree of peripheral vasoconstriction following either local or parenteral administration.

Effect on respiration. dl-β-Phenyl-*n*-propylmethylamine has a definite effect on the respiratory rate of normal unanesthetized dogs as well as in those anesthetized by the intravenous administration of sodium pentothal. In the former case the intravenous administration of 1 mgm of the amine produced a definite increase in the rate. In the latter experiments the administration of the compound in a dose of either 1 or 5 mgm. elicited a definite decrease in the respiratory rate, a slight increase in the excursions but practically no change in the oxygen consumption.

Aside from the effect on the respiratory rate and amplitude dl-β-phenyl-n-propylmethylamine also affects the bronchioles. Experiments were carried out on pithed dogs·using Jackson's chest plethysmograph. Following bronchoconstriction produced by arecoline hydrobromide, dl-β-phenyl-n-propylmethylamine in a dose of 5 mgm. produced a bronchodilatation (fig. 3). In a dose of 1 mgm., a similar but weaker effect was noted, less than that elicited by a similar amount of l-ephedrine.

Effects on the gastro-intestinal tract. The effect of dl-β-phenyl-n-propylmethylamine on the intestine was determined in some studies using isolated intestinal

Fig. 2

Fig. 3
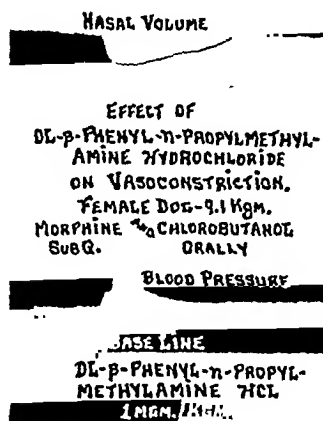
FIG. 2. VASOCONSTRICTOR ACTION IN DOGS

Simultaneous tracings of the pressor and vasoconstrictor effect of dl-β-phenyl-n-propylmethylamine, 1 mgm. Upper tracing is a recording of the nasal volume. A drop in the recording lever indicates an increase in nasal volume and an index of the degree of vasoconstriction.
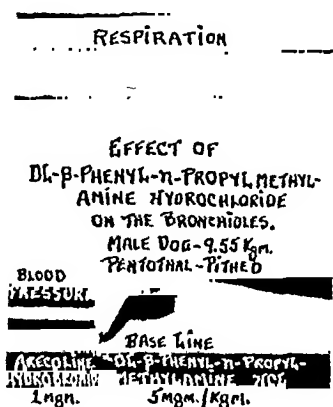
FIG. 3. BRONCHIAL ACTION IN PITHED DOGS

Bronchial dilatation produced by dl-β-phenyl-n-propylmethylamine, 1 mgm., following bronchial constriction elicited by arecoline hydrobromide, 1 mgm. (total dose).

strips. In a concentration of 1:5,000 both this compound and amphetamine produced a decrease in the tonus of the muscle, the action being slightly greater with amphetamine. Against acetylcholine or barium chloride-induced spasms the two compounds in 1:5,000 concentration produced a relaxation of similar intensity.

Some studies were also carried out on the intestine in situ in dogs either anesthetized with sodium pentobarbital or pithed. A balloon was inserted into the intestine and the movements recorded by means of a water manometer. Here again both amphetamine and dl-β-phenyl-n-propylmethylamine produced a relaxation of similar intensity.

Effect on the uterus. In these studies, the isolated uterus of both guinea pigs and rabbits was employed. Either amphetamine or dl-β-phenyl-n-propylmethyl-

amine in a concentration of 1 5 000 produced a very definite spasm of the uterine muscle There appeared to be very little difference in the degree of action of the two compounds

Effect on the pupil The mydriatic action of *dl β* phenyl *n* propylmethylamine following local application has been studied in rabbits A 1% solution of the compound was instilled into the conjunctival sac and allowed to remain there for one minute At the end of this period the excess solution was expressed Dilatation of the pupil appeared in about 20 minutes and reached its maximum at the end of about 30 minutes The light reflex was still present In some control experiments amphetamine in a 1% solution was applied in the same manner and was found to produce a greater degree of mydriasis than *dl β* phenyl *n* propyl methylamine

Central stimulation The central stimulant effect of this amine was determined and compared with that produced by amphetamine and *l* ephedrine The central stimulation was tested by measuring the total activity of white rats, using the method described by Schulte et al (2) All the compounds under test were administered orally in a dose of 50 mgm, and the results of these studies are presented in figure 4 These results include 12 animals for each compound, and 200 rats for the control values In figure 4 the abscissa gives the time in hours, the ordinate the average number of revolutions per hour It will be noted that amphetamine produced a very marked stimulation and reached its height in about 3 hours *l* Ephedrine also produced a marked degree of stimulation although not so great as that elicited by amphetamine *dl β* Phenyl *n* propyl methylamine produced no stimulation at this dosage level Following subcutaneous administration of 25 mgm also *dl β* phenyl *n* propyl methylamine failed to reveal any central stimulant action

DISCUSSION In their classical studies on the relationship of chemical structure to sympathomimetic action, Barger and Dale (3) concluded that the optimum constitution consisted of a benzene ring with a side chain of two carbon atoms of which the second bears the amine group Later work brought forth new evidence which necessitated the revision of this conclusion This work indicated that compounds with an isopropyl side chain may possess physiological actions which are greater than those of substances with only two carbon atoms A typical example of this type of compound is *l* ephedrine

 - Hartung and Munch (4) carried out some investigations to determine whether maximum activity could be obtained when the aryl and amino groups were separated by two aliphatic carbon atoms In their studies they compared the activity of four isomeric phenylpropylamines and their results indicated that Barger and Dale were substantially correct in their assertion that compounds should contain at least a β phenylethylamine skeleton if a rise in blood pressure is to be produced If this elementary structure is changed either by moving the amino group farther away from the phenyl or by moving it closer, the degree of physiological effect is greatly decreased Hartung and Munch have also noted that the substitution of a methyl group on either the α or β carbons conferred oral activity on the compound

Recently Proetz (5) reported the action of 2-amino-heptane sulfate as a nasal constrictor. This substance is of interest since unlike other constrictors it is an aliphatic compound. Chen and Swanson (6) have investigated its pharmacological properties. The activity of 2-amino-heptane sulfate in cats is about twice that of ephedrine and following intravenous administration to mice it is approximately three times as toxic. According to these investigators it is es-
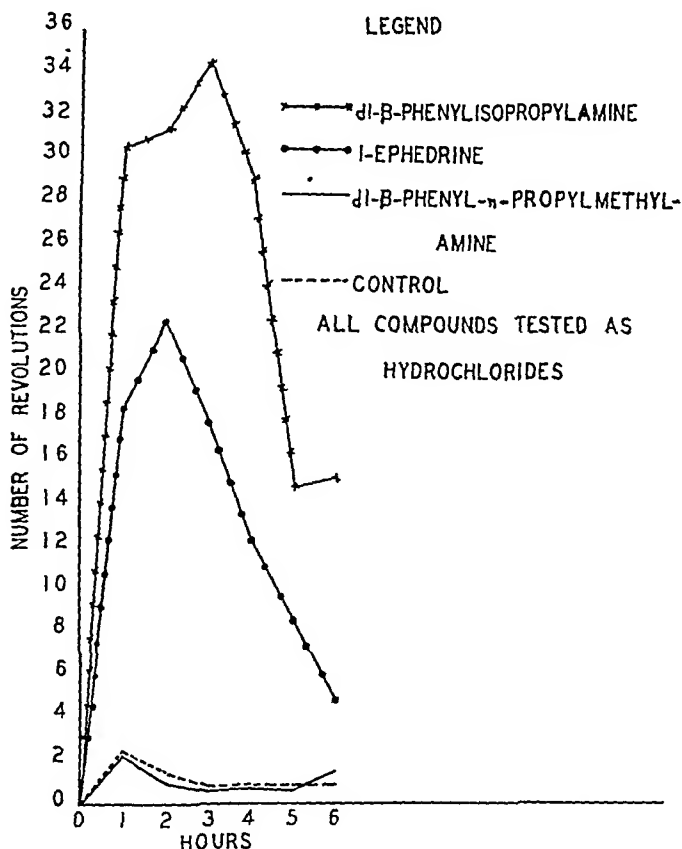
Fig. 4. Comparative Central Stimulant Action of *dl-β*-Phenyl-*n*-propylmethylamine, *l*-Ephedrine, and *dl-β*-Phenylisopropylamine

sential for the amino group to be attached to the second carbon in the chain to obtain a pronounced pressor effect.

The compounds in the present series may be considered as having the phenylethylamine skeleton. Table 1, compound no. 1, *dl-β*-phenyl-*n*-propylamine and no. 2, *dl-β*-phenyl-*iso*-propylamine, are two of the series reported on by Hartung and Munch. The pressor action of these compounds is the same; however, placing the methyl group on the β carbon considerably decreases the toxicity.

Compound no 8, *dl β* phenyl *n* butylamine, retains the two carbon skeleton, and has an ethyl group on the *β* carbon   With this increase of one carbon on the side chain the pressor action is definitely reduced   *dl β* Phenyl *β* methyl *iso* propylamine has a methyl group on both the *α* and *β* carbon atoms and this addition of a side chain reduces the pressor action   Compounds no 4 and no 5 have a methyl group on the *α* carbon and an ethyl and iso propyl grouping respectively on the *β* carbon   From the table it will be noted that with the increase in the side chain on the *β* carbon the pressor action is progressively decreased   If the *α* carbon contains two methyl groups as in *β* phenyltertiary butylamine, compound no 9, the original pressor action is retained and equal to that elicited by compounds no 1 and no 2   The toxicity is approximately the same as that of compound no 2   These results agree favorably with those obtained by Hartung and Munch, since the addition of a methyl group to either the *α* or the *β* carbon did not reduce the pressor action   Furthermore, these results indicate that a side chain on both the *α* and the *β* carbon diminishes the pressor action   This decrease is more marked as the length of the side chain is increased

Tainter (7) observed that *β 2* thienylethylamine has about the same pressor action in cats as *β* phenylethylamine   Burn studied the pressor effect of *β 2* thienylethylamine synthesized by Barger and Easson (8) and compared this with that elicited by *β* phenylethylamine and found that the results agreed both quantitatively and qualitatively   More recently Alles et al (9) noted the similarity in the pressor action of *dl β* phenyl iso propylamine and its thienyl analogues   These last two compounds are included in the present series, compounds no 2 and no 6   The pressor action was the same in both instances, but the toxicity of the phenyl derivative was somewhat greater than that of the thienyl derivative   This same relation exists between compounds no 3 and no 7

The compounds discussed thus far are primary amines with the skeleton structure of ethylamine, that is, with two carbons between the aryl and amino group   The results obtained with these compounds agree with or support the findings of Hartung and Munch, Tainter, Burn and Alles and his co workers

Various investigators have shown that primary amines have a greater pressor action than the corresponding secondary amines   Chen et al (10) demonstrated that phenyl ethanolamine has a greater pressor action than its corresponding methylated secondary amine, and that as the number of carbon atoms on the amino group is increased the pressor action is further reduced   This reduction in pressor action is also apparent when a secondary amine is converted to its corresponding tertiary amine   In the present series compound no 13 is the second ary amine of *β* phenylethylamine   This compound has a minimum pressor effect, much less than is elicited by the corresponding primary amine   However, a number of compounds in this series did not exhibit this decrease in pressor action following the conversion of a primary to a secondary amine   Compound no 10, the secondary amine of compound no 1, exhibits the same pressor action as the latter although the toxicity is markedly decreased   The same relation exists between the majority of the other compounds listed   For example compound no 12, the corresponding secondary amine of compound no 8, again retains the pressor action but the toxicity is decreased   It would appear, there

fore, that the type of compound having the skeleton of phenyl ethylamine with a side chain on the β carbon other than an OH group when converted to a methylated secondary amine does not have its pressor action decreased but does have the toxicity reduced. This type of compound, therefore, differs in this respect from phenylethylene or from β-phenyl ethanolamine. If the number of carbon atoms on the amino group is increased beyond the methyl group, or, if this type of compound is converted from a secondary to a tertiary amine, there is a very definite reduction in the pressor action. For example compound no. 12, the secondary amine of compound no. 8, did not lose any of its pressor action. However, when this was converted to a tertiary amine, compound no. 17, the pressor action was entirely lost and the compound actually exerted a depressor effect.

dl-β-Phenyl-*n*-propylmethylamine was selected from this series for more complete study for several reasons. This compound exerted as great a pressor action as amphetamine and yet was the least toxic of any of the substances eliciting the maximum pressor effect. Furthermore, this compound also had a minimum central stimulant effect. Besides possessing these desirable qualities, *dl*-β-phenyl-*n*-propylmethylamine is a volatile amine exerting 1 mm. vapor pressure at 65°C.

The relation of the toxicity as well as the pressor effect of this compound to the ᴄhemical constitution has already been discussed. In regard to its cardiac effects should be added that this substance, like amphetamine or *l*-ephedrine, has a ᴛachyphylactic effect following repeated or serial injections. Meek (11), in some ᴛudies on a series of compounds to be published later, studied the effect of *dl*-β-phenyl-*n*-propylmethylamine on the heart of normal dogs and on some anesthetized with cyclopropane. This compound was found to have little action as a cardiac excitant as measured by its ability to produce extrasystoles or ventricular tachycardia. These results fit in very well with those obtained in this laboratory on normal unanesthetized dogs.

dl-β-Phenyl-*n*-propylmethylamine definitely produces a bronchial dilatation of the same magnitude as that elicited by amphetamine. Since this effect is present in pithed animals it appears to be mainly a peripheral action. Alles and Prinzmetal (12) compared the bronchial actions of *dl*-β-phenylisopropylamine and β-phenylethylamine with their pressor effect and found that these two types of actions paralleled each other in these compounds. The results of the present study indicate that this parallelism also exists between *dl*-β-phenyl-*n*-propylmethylamine and *dl*-β-phenylisopropylamine.

The decrease in the central stimulant action apparently is due to the change from an isopropylamine to a *n*-propylamine structure. It will be recalled that Schulte et al. (2) found that the *d*-phenylisopropylamine structure gave the greatest central stimulant action. Any deviation from this type of structure was accompanied by a marked decrease in the stimulant action.

dl-β-Phenyl-*n*-propylmethylamine thus differs from amphetamine in that it is much less toxic than the latter and that it evokes a minimum, if any, central stimulant effect. Like amphetamine it is a volatile amine and exerts a similar pressor and vasoconstrictor action. Since *dl*-β-phenyl-*n*-propylmethylamine is

relatively non toxic and elicits a minimum of central stimulation it may prove useful clinically in the treatment of various types of nasal congestion

SUMMARY

A series of aryl ethyl , aryl propyl , and aryl butylamines were synthesized and tested in a preliminary manner in an attempt to find a volatile amine which would possess a good vasoconstrictor action, elicit a minimum degree of central stimulation and possess relatively low toxicity Of this series *dl β* phenyl *n* propylmethylamine, a volatile amine, appeared the most promising in respect to toxicity and pressor action

The toxicity of this compound is of the same order as *l*-ephedrine and about one half that of amphetamine

The effect on the cardiovascular system and on the bronchioles is very similar both quantitatively and qualitatively to that elicited by amphetamine This compound produces very little if any central stimulation in the experimental animal

The relation of the pharmacological action of *dl β* phenyl *n* propylmethyl amine to its chemical structure has been discussed

Results of studies on a series of compounds indicate that a compound having the skeleton of phenylethylamine with a side chain on the *β* carbon other than an OH group when converted to a methylated secondary amine does not alter its pressor action decreased but does have the toxicity reduced

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EFFECT OF ORAL ADMINISTRATION OF SÚCCINYL SULFAPY-RAZINE ON BACTERIAL (COLIFORM) FLORA OF THE INTESTINE OF NORMAL MICE

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The reproduction of human intestinal diseases, such as typhoid fever and bacillary dysentery has not yet been adapted to work in experimental animals. Experimental investigation of the chemotherapeutic activity of the sulfonamides against these infections would be less difficult if it were possible to reproduce the intestinal symptoms of these diseases in laboratory animals. Experiments in vitro cannot be compared with the conditions existing in the human intestinal tract and therefore do not give a satisfactory basis for the evaluation of drug efficacy.

Marshall, Bratton, White and Litchfield (1) developed a method for evaluating the bactericidal activity of the sulfonamides by determining the effect of sulfaguanidine on the normal intestinal (coliform) flora of the laboratory mouse. Counts of coliform bacteria in fresh stool specimens of normal mice were made on desoxycholate agar plates. This method represents an indirect means of determining the density of these bacteria in the contents of the intestine.

The compound sulfanilylguanidine ("sulfaguanidine"), which is fairly water-soluble, showed bactericidal effect, but only low absorption from the intestinal tract of animals. Other investigators (2) found a marked bacteriostatic action on the coliform organisms in both the animal and human intestinal tract after administration of succinyl sulfathiazole ("sulfasuxidine"). Such observations stimulated investigation of the efficacy of other sulfonamide derivatives. Recently H. White (3) determined quantitatively the comparative activity in mice of various sulfonamide compounds, such as sulfapyrazine, sulfadiazine, sulfathiazole, sulfaguanidine, succinyl sulfathiazole, etc.

In human therapy, the results obtained with sulfaguanidine and succinyl sulfathiazole in the treatment of bacillary dysentery have been particularly encouraging (4–11). G. M. Lyon (9) recently stated: "Chemotherapy has revolutionized the treatment of bacillary dysentery". However, the respective values of these two compounds in human therapy are not yet sufficiently clear. Judging from the literature, there is some disagreement concerning the relationship between their bactericidal effectiveness and there potential toxic effect. As to the other sulfonamide derivatives tested in normal mice, the extent of their use in the

therapy of bacillary dysentery is as yet too small to permit an estimate of their value.   After all, the need for a compound which can be administered to human beings in a dose great enough to produce the highest possible effect *in loco* and yet be as slowly and slightly absorbed as possible from the intestine, makes further chemotherapeutic investigation necessary.

Investigating a series of new compounds developed at the Dermatological Research Laboratories, Philadelphia, Division of Abbott Laboratories, North Chicago, Ill, we found that product #3163 provided encouraging data for further investigation.   In this paper we limited our report to the results obtained with this new compound, adding only one other product of the same series, i e , #3157, which was included in the first experiment of this investigation   The bactericidal effectiveness of both compounds in normal mice was compared with the action of sulfaguanidine and succinyl sulfathiazole.

Product no 3163—succinyl sulfapyrazine was obtained by the interaction of sulfapyrazine and succinic anhydride   It has the following structural formula.

$$COOH—CH_2—CH_2CONH \diagdown \diagup —SO_2NH \diagdown \diagup$$
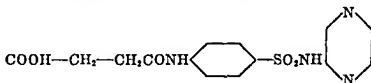
In the above chemical compound the hydrogen in the amino group of the sulfapyrazine was replaced by a carboxyl of the succinic acid   The remaining carboxyl permits the formation of the sodium salt, so that the product is soluble in sodium bicarbonate as well as in sodium carbonate and sodium hydroxide   It is soluble in hot water and crystallizes on cooling   The product used in these experiments was recrystallized and according to analysis proved to be a pure chemical compound   It is insoluble in dilute hydrochloric acid

Product no 3157—malyl sulfathiazole was obtained by the interaction of malic acid and sulfathiazole   It has the following structural formula.

$$HC—S$$
$$HC \quad C—NH—SO_2— \diagdown \diagup —NH—CO—CH_2—CHOH—COOH$$
$$N$$
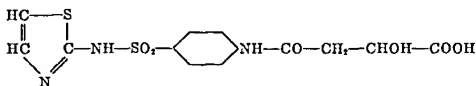
The pure compound was used in these experiments   The above product is soluble in sodium hydroxide, sodium carbonate and sodium bicarbonate   It is insoluble in dilute hydrochloric acid and very slightly soluble in water

CHRONIC TOXICITY AND BLOOD CONCENTRATIONS.   In the first series of experiments five mice received daily five grams of succinyl sulfapyrazine per os in 10 per cent gum acacia suspensions   The above amount was administered daily to each mouse for ten days consecutively.   The total amount received by each animal was 50 grams.   The mice were observed for eight days after the drug was discontinued.   All the animals survived   The identical procedure was employed in the case of five other mice when each animal received the same total amount of 50 grams of sulfaguanidine.   All mice survived the period of ten days of drug administration and the eight days of subsequent observation.   The

third group of five mice received sulfasuxidine, again each receiving a total of fifty grams in ten days. These animals likewise survived the period of eighteen days. The above experiments indicate that succinyl sulfapyrazine, sulfaguanidine and sulfasuxidine (succinyl sulfathiazole) are tolerated in large single doses when given by mouth to mice.

In the second series of experiments we worked with three chemical compounds in which we were particularly interested because of their bactericidal activity. We mixed these compounds thoroughly with food and fed them to mice. As estimated from weighings of the food consumed, the animals ate daily approximately 5 grams containing 5 per cent of the drug. On the first day, the mice ate less than subsequently but from the second day to the last (or tenth) day, a constant intake of about 5 grams was observed—this amount containing 5 per cent or 250 milligrams of the pure drug. Except for the death of one animal receiving sulfaguanidine, no other deaths occurred among the 15 mice, which were divided into three groups—each consisting of 5 mice that received either succinylsulfapyrazine, sulfaguanidine, or sulfasuxidine.

In the third experimental series, blood levels were determined in 10 mice that received food containing 1 per cent of the drug. Of the four mice examined in this group to which succinylsulfapyrazine was administered, one animal showed 3.8 milligrams of the drug (free sulfapyrazine) per 100 cubic centimeters of blood; another was found to contain 3.2 mgm. per cent of free and 2.1 mgm., per cent of conjugated sulfapyrazine; the third animal had 4.8 mgm. per cent of the free and 2.9 mgm. per cent of the conjugated drug; the fourth mouse had 3.8 mgm. per cent of free and 1.3 mgm. per cent of conjugated sulfapyrazine. Of ten mice given food with one per cent sulfaguanidine, one showed 0.9 mgm. per cent sulfaguanidine; the second had a blood concentration of 1 mgm. per cent free and 0.7 mgm. per cent conjugated drug; in the third animal was found 1.2 mgm. per cent of free and 0.5 mgm. per cent conjugated drug; the fourth animal had 1.4 mgm. per cent of free and 0.3 mgm. per cent conjugated sulfaguanidine. In another experiment 10 mice were fed with food containing 1 per cent sulfasuxidine. One mouse had 0.2 mgm. per cent of free and 2.3 of conjugated drug; the second animal showed 0.6 mgm. per cent free and 0.7 conjugated drug; the third mouse showed 0.9 mgm. per cent of the free and 0.4 mgm. per cent of the conjugated drug; the fourth animal was found to have 1.1 mgm. per cent of free and 0.6 mgm. per cent of conjugated sulfasuxidine.

All animals not killed for the purpose of taking the blood, survived a period of 18 days. We assume that as a result of the absorption of succinyl sulfapyrazine from the intestines, it appeared in the blood as a free compound, and also in a conjugated form, probably acetyl sulfapyrazine. With a comparatively large dose such as 1 per cent of the drug in food, moderate quantities of the free or the combined drug appeared in the blood. Blood levels for sulfaguanidine and sulfasuxidine (succinyl sulfathiazole) were found to be lower. In view of the excellent toleration of succinyl sulfapyrazine, when given in very large quantities to mice by mouth, the amount of the drug present in the blood stream was low enough to prevent any increase in the chronic toxicity.

EXPERIMENTAL   The method published by Marshall, Bratton, White and Litchfield (1) gave the basis for our experimental technique

White mice of 18 to 23 gms of weight were used   Twenty to 25 mgms of fresh stool specimens[1] were taken directly from each mouse by a flamed inoculation loop and emulsified in 2 cc of broth   The formed pellets were broken up and stirred into an emulsion by means of a heavy metal inoculation loop which was flamed before and after use with the specimen of each mouse   A small sterile cotton pad was placed in the mouth of each sterile test tube and was pushed down through the emulsion in order to retain gross particles and to make the suspension as uniform as possible   Pour plates containing 20 cc of desoxycholate agar (Leifson) were made with 1 cc amounts of a 1 100 broth dilution of each original emulsion   According to the method described by Leifson (12), colony counts were made after incubation at 37°C   In the majority of cases the results were obvious after only 18 to 20 hours when the significant red colonies of coliform organisms were in rapid development and colonies of other bacteria were generally still invisible or visible as very small colorless colonies   The type of colony which we counted also showed the other morphological and cultural characteristics of coliform bacteria

First experiment   After having been fed in individual cages with the normal diet for eight days, six animals were selected for each of the compounds , i e , 24 mice, divided into four groups   Each group was put on 1 per cent drug diet for eight days 6 mice on 1% sulfaguanidine diet, 6 mice on 1% succinyl sulfathiazole diet 6 mice on 1% diet prepared with product no 3157, 6 mice on 1% diet prepared with product no 3163

For normal diet we used Wayne Fox Chew   This food was pulverized and mixed with drug   After cessation of drug diet the normal food was re administered for another eight day period   The average daily consumption of food was 5 gms per mouse   By weighing the eventual residue, the daily food intake and the respective drug intake could be determined for each mouse   In general, the animals became accustomed to drug diet quickly   The intake was uniform from the third day when the daily intake of compound averaged 05 gm per mouse   Only two mice (no 4 of the succinyl sulfathiazole group and no 3 of the no 3157 group) which never consumed more than 3 8 to 4 5 gms of food per day, averaged 033 to 045 gm of compound daily

At least three counts were made on each mouse before beginning drug diet, three counts during drug diet and another three counts after cessation of drug diet and readministration of normal diet   We determined the average numbers of bacterial colonies from the second and third counts of each eight day period of feeding when the food intake had become uniform   These determinations were made on the fifth and the seventh days

As did H White (2), we found that even under the same conditions of care and feeding the number of coliform colonies is not at all uniform in normal mice   Also, in our experiments occasionally one mouse did not show coliform colonies when stool specimens were cultured on desoxycholate agar   In one mouse which we observed through six weeks the cultures showed only aerobacter colonies in twelve counts   In several other mice which had coli form bacteria in the feces, daily changes were observed in the number present although the fecal pellets did not show any change

In selecting the animals before starting the chemotherapeutic experiment, we eliminated all mice which had shown less than a thousand colonies in two tests immediately preceding the period of drug administration   The presence in the normal feces of a thousand to innumerable colonies gave a substantial basis for evaluating the later effectiveness of the drugs in that a marked change could be observed from thousands or innumerable colonies present in normal feces, to less than a hundred colonies during drug diet   In the majority of cases the bacteriostatic action against the coliform bacteria became obvious after only 48 hours   However, the greatest bactericidal effect was reached on the fourth day of drug intake   Generally aerobacter colonies, characterized by pink color or red centers and

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[1] Average weight of fresh fecal pellets equaled 0235 gms (determination made on four days with eight mice fed with normal diet)

colorless peripheries, proved to be much more resistant against the drugs than the coliform bacteria. This selection of animals required a great many preliminary tests before starting the drug diet because no more than approximately 60 per cent of the normal mice of our stock showed a standard count of a thousand or innumerable coliform colonies during the eight-day period of the pre-treatment counts. All mice tolerated the eight-day period of drug administration well.

Results. The tabulated results (table 1) are self-explanatory. The symbols H, T and M indicate colony counts of hundreds, thousands or innumerable ("millions") colonies while the tabulated figures refer to the actual numbers of colonies, all symbols indicating the average of two counts made on the fifth and seventh day of each feeding period. In comparing the various results, one is at once cognizant of the superiority of the bactericidal action of sulfaguanidine and

TABLE 1

*First experiment: effect of one per cent drug diet on the colony count of the intestinal (coliform) flora of normal mice (desoxycholate agar plate count)**

	COMPOUND											
	Sulfaguanidine						Succinyl sulfathiazole					
	Mouse number											
	1	2	3	4	5	6	1	2	3	4	5	6
Before drug diet.....	M	M	T	T	M	M	T	T	T	M	T	M
During drug diet.....	12	90	0	83	30	90	H	H	H	M	16	T
After drug diet.......	H	T	T	T	T	H	T	M	T	M	T	T
	#3157 Malyl sulfathiazole						#3163 Succinyl sulfapyrazine					
Before drug diet.....	T	M	M	M	M	M	T	T	M	T	T	T
During drug diet.....	H	H	M	31	40	H	0	21	0	30	15	6
After drug diet.......	H	M	M	H	T	T	T	T	38	T	T	H

* The noted symbols or figures indicate the average number of colonies counted on the fifth and the seventh day of each eight-day period of feeding: H = hundreds T = thousands M = innumerable ("millions").

product no. 3163 (succinyl sulfapyrazine) over the other two drugs tested. In all mice used for either compound a rapid decrease in the development of coliform colonies was obvious, such as a change in the original average of M or T to less than a hundred colonies. In one mouse of the sulfaguanidine group and in two of the no. 3163 group, coliform bacteria proved to be reduced to such a degree that these organisms could not be demonstrated by growth on desoxycholate agar. Comparatively, in none of the animals fed with the other two compounds was there such a significant effectiveness. Only two mice of the no. 3157 group gave average results below a hundred colonies. We cannot explain the apparent lack of any effect in another mouse of both the no. 3157 and the succinyl sulfathiazole group (no. 4 of the latter and no. 3 of the no. 3157 group), since the original counts of M colonies remained constant during and after drug diet. But since both animals belonged to the few whose daily food intake remained lower during

the whole experiment than that of the other mice, the somewhat lower drug intake may have been responsible   In several mice of all compound groups the effect of drug diet was so lasting that the number of coliform bacteria in the feces never returned to the original T or M counts during the observation period of our experiment

Second experiment   In the second experiment we attempted to substantiate the results attained in the first with product no 3163   The same procedure was followed, using a series of 20 normal mice with average counts of T or M coliform colonies   Administration and concentration of drug diet and all other factors were the same as those of the preceding experiment

Results   The results tabulated on table 2 demonstrate again the high bactericidal action of product no 3163 against the intestinal bacteria of normal mice   Also in this series 1 per cent drug diet was well tolerated   Only two mice (no 12 and no 25) showed an average count of more than a hundred coliform colonies after drug administration "   The other 18 mice averaged from zero counts

TABLE 2

Compound #3163 Succinyl sulfapyrazine—second experiment—20 animals

	MOUSE NUMBER																			
	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Before drug diet	M	T	T	T	T	M	M	T	M	M	M	M	M	M	M	T	T	T	M	T
During drug diet	0	20	2	33	18	H	30	35	36	0	94	70	0	19	85	32	63	45	T	43
After drug diet	M	90	H	T	H	T	T	T	T	H	M	H	H	42	H	T	T	H	T	H

Explanation of symbols and figures the same as given for table 1

(three animals) to 94 colonies   Nearly half of the animals showed a marked reduction in density of coliform bacteria in the stools which remained until the end of our observation period   In three of these mice we continued observation for another 8 day period, the one (no 9) showed remarkable increase of the coliform colonies only on the thirteenth day after drug administration was stopped, the other mice (no 8 and no 19) showed a constant low number of colonies even two weeks after cessation of drug intake

Discussion   In comparing the results obtained with the various compounds in our first experiment, we found a marked bactericidal effectiveness of product no 3163 (succinyl sulfapyrazine) against the intestinal coliform bacteria of normal mice   This compound proved at least equal to sulfaguanidine and considerably superior to succinyl sulfathiazole (sulfasuxidine)   The product no 3157 (malyl sulfathiazole) proved about equal to succinyl sulfathiazole under the conditions of our experiment   H White (3), in his quantitative evaluation, also found the bactericidal effectiveness of sulfaguanidine better than that of succinyl sulfathiazole, the highest degree of activity among the ten sulfonamides

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² Mouse no 25 was the only one showing a constant lower intake of drug diet as compared with the other mice of this series   Therefore the drug concentration in the stool specimens of this animal apparently was not sufficient to diminish the numbers of colonies

which were tested in his investigation was observed after administration of sulfapyrazine. Product no. 3163, which proved highly effective in our series, is succinyl sulfapyrazine.

The results obtained in our second experiment, using an additional twenty normal mice, substantiated the high bactericidal activity of succinyl sulfapyrazine. Combining the results obtained in the first and second experiments, we find a marked constancy of the low figures of colonies observed during the administration of succinyl sulfapyrazine. Only two of the total of 26 animals which were treated with this compound in both experiments showed an average of more than a hundred colonies (= about 8%), while 24 animals (= 92%) demonstrated a decrease from T or M pre-treatment counts to less than hundred colonies, or—in four mice—to zero colony counts. In comparing this comprehensive result with the colony counts after one per cent. sulfaguanidine diet which Marshall and his co-workers tabulated in their first publication on sulfaguanidine (1), we find again at least an equal effectiveness of product no. 3163 (succinyl sulfapyrazine) and sulfaguanidine.

After the 8-day period of drug diet, in some animals we observed a lasting change of the intestinal flora up to the end of the observation period, particularly after succinyl sulfapyrazine, in that the original counts of T or M colonies never again were reached. In his experiments, H. White (3) observed that a marked bactericidal effect of a 5-day treatment with one per cent. sulfaguanidine diet did not last long after cessation of drug administration. However, in a great many of our animals the coliform counts also gradually increased to the pre-treatment counts of T or M colonies after cessation of drug intake.

SUMMARY

The bactericidal action of two new sulfonamide derivatives, i.e., succinyl sulfapyrazine and malyl sulfathiazole, against the intestinal coliform flora of normal mice was determined and compared with the action of sulfaguanidine and sulfasuxidine (succinyl sulfathiazole). Under the conditions of our experiments, succinyl sulfapyrazine proved highly effective and at least equal to sulfaguanidine in diminishing the density of coliform bacteria in the contents of the intestine of normal mice. Its bactericidal effectiveness was much greater than that of sulfasuxidine and greater than that of malyl sulfathiazole. This last compound proved about equal in its effectiveness to sulfasuxidine.

The toxicity of succinyl sulfapyrazine, sulfaguanidine and sulfasuxidine (succinyl sulfathiazole) proved to be very low for mice when administered in large quantities in acacia suspension or in a drug diet. The resulting blood levels in mice on one per cent. drug food mixture were generally low for all three drugs, with succinyl sulfapyrazine somewhat higher than the other two drugs.

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THE PERIPHERAL CIRCULATORY ACTION OF VERATRUM VIRIDE

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Veratrum viride is obtained from the rhizomes and roots of the green hellebore. The presence of the three alkaloids jervine, pseudojervine and rubijervine is well established (Wright (1)) but they are relatively inactive biologically (Boehm (2)). Although Wright presented evidence that cevadine (crystalline veratrine), the important alkaloid of Veratrum sabadilla, was present in relatively high concentrations, he did not definitely establish this and it has not yet been confirmed. Seiferle, Johns and Richardson (3) were unable to find in Veratrum viride, protoveratrine (Salzberger (4)) and germerine (Poethke (5)) the highly active alkaloids of the closely related species Veratrum album. They did isolate, however, protoveratridine and germine which are hydrolysis products of germerine. Their results also showed the presence of definitely toxic alkaloids which they were unable to identify. Consequently the principle or principles primarily responsible for the activity of the drug are still not established. The similarity of the descriptions in the literature, however, of the circulatory effects of Veratrum viride, Veratrum album (including protoveratrine and germerine) and even of veratrine suggest that these drugs contain closely related principles.

Despite the fact that the first physiological investigations of the action of Veratrum viride were carried out over 100 years ago, relatively little is known of its effects on the vascular system. During the latter part of the 19th century the drug was used in the treatment of pneumonia, fevers and eclampsia because its administration was followed by a decrease both in the rate and in the force of cardiac action. After the extreme toxicity of large doses was recognized Veratrum was prescribed less frequently; its use was continued, however, by a few individuals in the treatment of the convulsive toxemias of pregnancy. Clinically Veratrum viride is used in the form of the tincture or as Veratrone,[1] a nonalcoholic preparation which may be given parenterally. The recent report by Bryant and Fleming (6) of 120 cases of eclampsia treated with Veratrum viride with a mortality rate of only 1.6% warrants further investigation of its use as an adjunct treatment for this disease. Because of its powerful depressant effect on both pulse rate and blood pressure it seems desirable that the physiological effects of the drug be investigated more thoroughly before its general use is advocated.

The outstanding effect of moderate doses of Veratrum viride injected intravenously in animals is a fall in blood pressure resulting primarily from cardiac

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[1] Veratrone, a preparation of Parke, Davis and Company is described as "a purified preparation of Veratrum Viride, equal to one-fourth the strength of the Fluid Extract by alkaloidal assay."

slowing due to vagal stimulation. The role of vasodilatation in contributing to the fall in blood pressure has received comparatively little attention although reference has been made to it by various investigators. MacNider (7) observed some fall in blood pressure on intravenous injection of the tincture into vagotomized or atropinized dogs and also a reduction of the hypertension produced by epinephrine which could not be accounted for by the slowing of the heart. Cramer (8), using cats, concluded that, although cardiac slowing contributed to the fall in blood pressure, the latter was due primarily to vagal reflex vasodilatation. An increase in intestinal volume and a temporary decrease followed by an increase in kidney volume were also observed. The vasodilatation was not believed to result from a peripheral action since epinephrine and posterior pituitary extract still produced the usual degree of vasoconstriction. On the contrary, Hewlett (9), using an arm plethysmograph on human subjects, concluded that the fall in blood pressure was related to cardiac slowing rather than to vascular change.

Following the injection of Veratrone in a patient being treated for convulsive toxemia of pregnancy at the University of Michigan Hospital a marked dilatation of the retinal arterioles coincident with the fall in blood pressure was noted. This observation plus the fact that there often is a distinct improvement in the general condition of the toxemic patient after the use of Veratrum suggested that factors other than depression of the pulse rate and blood pressure might be concerned. *This study was undertaken in an effort to determine the action of the drug on the peripheral circulatory system.*

METHODS. Three types of experimental procedure were used.

1 *Perfusion of isolated organs.* The main artery and vein of the isolated ear, kidney and hind leg of the rabbit were cannulated and the vascular system was perfused with mammalian Ringer's solution at constant pressure. Each drop of fluid emerging from the *vein was recorded by means of a drop counting device. After the establishment of a regular* rate of flow the drug was injected by a small gauge hypodermic needle into the artery and the effects on the perfusion rate observed.

2 *Studies on intact animals.* Dogs under morphine urethane anesthesia were used. The blood pressure was recorded by a mercury manometer from the carotid artery and volume changes in the spleen and hind leg were recorded by plethysmographs. The drug was administered directly into the femoral vein usually in dosages of 0 005 cc (Veratrone) and 0 02 cc (tr Veratrum viride) per kgm of body weight.

3 *Studies on animals in which the peripheral circulation was excluded.* Dogs anesthetized with morphine and urethane were used. The subclavian vessels were clamped bilaterally at their origins and the aorta was clamped just below the arch thus eliminating the peripheral circulation and leaving intact the circulation through the heart lungs and head. Artificial respiration was begun on opening the chest wall. The drug was administered into the jugular vein and the effect on carotid blood pressure was recorded by means of a mercury manometer.

RESULTS 1 *Perfusion of isolated organs* (fig 1)

(a) Kidney. Studies on the perfusion rates through 25 kidneys were made. In each experiment the drug was injected into the cannula after the perfusion rate had become constant. In 9 instances the injection of the Veratrone was

preceded by epinephrine to constrict the vessels; in the remaining 16 only Veratrone was used.   The drug was injected in dosages varying from 0.005 to 0.1 cc.

The perfusion rate following the injection of epinephrine was markedly slowed as the calibre of the vessels decreased but when this drug was followed by Veratrone there occurred in each instance a distinct and rapid increase in the rate of flow.   In the remaining 16 experiments an increase in flow was noted in 12 (dosage 0.01–0.05 cc.).   The amount of the drug injected in the 4 which failed to respond was only 0.005 cc.; the perfusion rate in these organs increased with a larger dose.   An increase in perfusion rate never occurred with a control injection of an equal volume of Ringer's solution.
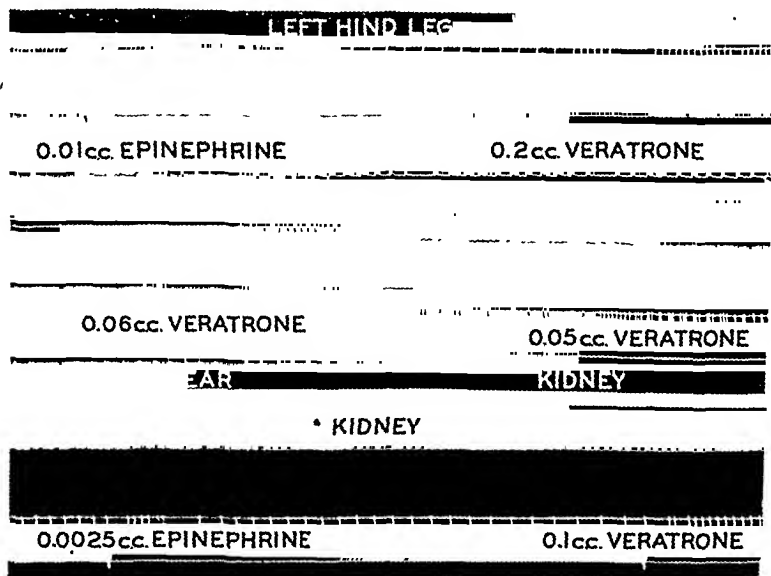
FIG. 1. EFFECT OF VERATRONE ON THE RATE OF PERFUSION THROUGH ISOLATED STRUCTURES
OF THE RABBIT
Time record, 5 seconds

(b) Ear. Studies were done on 29 ears. In 9 the Veratrone was preceded by epinephrine, and in 20 only the former drug was given. The dosage of Veratrone varied from 0.001 to 0.08 cc. The perfusion rate through the vessels constricted with epinephrine was increased uniformly by the administration of Veratrone. In 19 of the remaining 20 ears the rate of flow increased when the drug (0.05–0.08 cc.) was given. The dosage in the ear which failed to respond was only 0.001 cc. No change in perfusion rate was noted when Ringer's solution was injected.

(c) Leg. Studies were made on 19 isolated legs in which all the circulation except that through the femoral artery and vein was occluded. In 15 legs the perfusion rate was increased by the injection of Veratrone (0.05–0.5 cc.) while

in the remaining 4 no change was noted The dosage in those which failed to respond varied from 0 005 to 0 01 cc No change in perfusion rate followed the injection of comparable volumes of Ringer s solution

2 *Studies on intact animals* In 12 dogs Veratrone was injected into the femoral vein prior to vagotomy and the blood pressure and spleen and leg volumes were recorded In all there was noted a definite depression of the pulse rate, in three instances the cardiac action was inhibited completely until relieved by section of the vague nerves Almost immediately following the first evidence of slowing of the pulse there occurred a sharp drop in blood pressure which was then maintained at a level lower than the initial pressure for from one to three hours if the vagi were allowed to remain intact Associated with the fall in blood pressure and occurring simultaneously with it an increase in leg volume was noted in each animal The change in most instances was evidenced by a sharp rise which was maintained at a level higher than the original for an average period of 5 6 minutes The change in spleen volume was less definite In
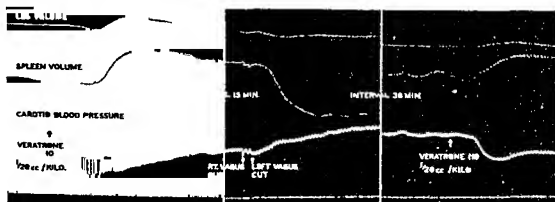
FIG 2 EFFECT OF VERATRONE ON AN 11 5 KG ANESTHETIZED DOG
Time record 5 seconds

half the animals there occurred an increase and in the remaining half a slight constriction of that organ (fig 2)

Section of the vagus nerves immediately restored the pulse rate to normal and resulted in an increase in the blood pressure although it rarely returned to the original preinjection level

In 6 animals the vagus nerves were sectioned prior to the injection of the drug In each one, following the administration of Veratrone there occurred a distinct fall in blood pressure without demonstrable change in the pulse rate The fall in blood pressure was less than that which occurred in animals with intact vagus nerves An increase in leg volume was noted constantly and in 4 of the 6 dogs there was also a definite increase in the volume of the spleen The initial drop in blood pressure was followed by a slight increase but in all animals the pressure was maintained at a level lower than that before the injection In the 12 animals to which veratrone was given before vagotomy the first dose of the drug after section of the nerves was followed by a fall in blood pressure and an increase in leg volume in all instances and in 6 animals an increase in spleen volume occurred (fig 2)

A total of 25 injections of veratrone were given to the 18 dogs subsequent to the first dosages after vagotomy. In each instance there occurred a rise in blood pressure above the pre-injection level which was maintained for an average of 2.3 minutes. The pressure returned to the original level after 5 of the injections and to a lower level after the remaining 20. No change in pulse rate occurred with the blood pressure fluctuations. A slight increase in leg volume was observed each time but the spleen increased in size in only 4; in the remainder there occurred a sharp contraction. The respiration became very irregular and labored after 11 of the injections and one animal died of respiratory failure.

3. *Peripheral circulatory exclusion.* In the 4 dogs in which the peripheral circulation had been excluded the injection of large doses of Veratrum viride (0.1 cc per kgm.) into the jugular vein was followed immediately by complete

TIME 5 SECONDS

CAROTID BLOOD PRESSURE

VAGUS NERVES CUT

TR. VER.
VIR. 0.5 cc.

TR. VER. VIR
0.5 cc.
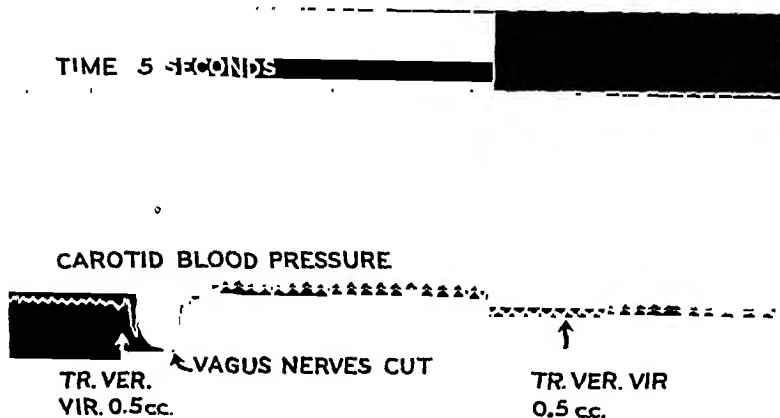
FIG. 3. EFFECT OF TINCTURE OF VERATRUM VIRIDE ON A 5.5 KG. ANESTHETIZED DOG, BEFORE AND AFTER VAGOTOMY WITH THE PERIPHERAL CIRCULATION EXCLUDED

suppression of cardiac action and a marked fall in blood pressure. Following section of the vagus nerves the pulse rate returned to normal and the blood pressure to a level slightly higher than the preinjection pressure. Subsequent injections of the drug resulted in no change in either pulse rate or blood pressure and no gross cardiac irregularities could be detected (fig. 3).

DISCUSSION. The fall in blood pressure following the injection of Veratrum viride in the anesthetized dog is primarily a result of vagal inhibition of cardiac action which masks the fall in blood pressure due to peripheral vasodilatation. A definite peripheral vasodilating action is demonstrated by the following experimental results: (1) the increase in perfusion rate in isolated organs, (2) the fall in blood pressure without demonstrable change in heart rate and with increase in volume of the leg and spleen in vagotomized animals, (3) the persistence of a blood pressure below the preinjection level in most cases after vagotomy in spite of the fact that the pulse rate again became normal. The increase in leg

volume and in about half the cases of spleen volume after injections with vagi intact is also in keeping with a peripheral vasodilatation. However, little weight can be placed on this observation alone since an increase in volumes of the spleen and leg may be due in part to venous congestion as a result of cardiac slowing. Cramer (8), using Veratrum viride in cats concluded that the fall in blood pressure due to peripheral vasodilatation was dependent on afferent vagal impulses. Jarisch and Richter (10) reached the same conclusions with respect to veratrine in cats. The evidence of peripheral vasodilatation observed by us in isolated structures of the rabbit and in vagotomized dogs shows that afferent vagal reflexes are unnecessary for its occurrence.

A direct depressing effect on cardiac muscle by large doses of Veratrum has been postulated by Houghton and Hamilton (11). Wedd and Drury (12) noted that the administration of the drug to patients with auricular fibrillation produced a reduction in both auricular and ventricular rates. Since in this condition vagal stimulation usually is followed by a decrease in ventricular rate and an increase in auricular rate they assumed that a direct effect on the auricle with an increase in the A-V conduction time was responsible. In their animal experiments they could detect little change in the normal heart unless extremely large doses were used. In our experiments in which the peripheral circulation was excluded, doses of the tincture five times as great as those used in the intact animal produced no perceptible effect on the heart or blood pressure after vagotomy.

The present work offers no explanation for the rise in blood pressure commonly observed, especially in vagotomized animals on repeated dosage with Veratrum and veratrine alkaloids. Although Pilcher and Sollmann (13), using a specialized technique, obtained little evidence of stimulation of the vasomotor center with Veratrum viride it seems probable that such occurs. Central vagus stimulation, convulsions with large doses even with artificial respiration and respiratory effects all of which have been described by other investigators point to a central action which by inference might involve the vasomotor center. Although reflex action is not excluded, vasomotor center stimulation is suggested by our experiments in that no appreciable alteration in heart action could be detected coincident with the rise in blood pressure and in our perfusion experiments a peripheral vasoconstriction was never observed.

SUMMARY AND CONCLUSIONS

1. In perfusion experiments on the isolated ear, kidney and leg of the rabbit a vasodilating action of Veratrum viride was clearly demonstrated.

2. Evidence of vasodilatation was obtained in that Veratrum viridi produced, in the anesthetized vagotomized dog, a fall in blood pressure and an increase in the volumes of the leg and spleen without perceptible change in cardiac action.

3. With the peripheral circulation excluded exceptionally large doses of the drug had no effect on cardiac action after vagotomy ruling out a direct depression of the heart.

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STUDIES ON VERATRUM ALKALOIDS[1]
IV THE SITES OF THE HEART RATE LOWERING ACTION OF VERATRIDINE

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The heart rate lowering action of veratrum alkaloids has been the subject of various studies which have led to apparently controversial results with regard to the mechanism involved in this action   In the following report we restrict ourselves to a discussion of the effect of veratrine (which is a mixture of alkaloids from the seeds of *Veratrum Sabadilla Aiton*, or *Schoenocaulon officinale Gray*), and especially of the pure alkaloids veratridine, cevadine, and cevine   The alkaloids of *Veratrum viride* and *Veratrum album* will be dealt with later

Von Bezold and Hirt (1) were the first to observe the heart rate decrease after veratrine   They used rabbits and cats for their experiments, and they explained the effect as being caused by a central vagal stimulation   Lissauer (2) using cevadine in rabbits and cats, did not consider this explanation to be definitely established, while Pilcher and Sollmann (3), on the basis of experiments in dogs, called attention to the value "of veratrum and cevadine as illustrating pure central vagus stimulation "

In cross circulation experiments in dogs Heymans and Heymans (4) found a decrease in heart rate when veratrine was injected into the arterial supply of the head which was perfused from another dog and had only the vagal connections with the heart   Their results would be in agreement with the above mentioned view unless afferent impulses originating in receptor areas which were included in the head circulation, like those of the carotid sinus were in part or wholly responsible for the effect   Later experiments conducted in Heymans' laboratory support the assumption that the carotid sinus area plays some role in the action under discussion [see Jarisch and Richter (5), page 348]

Jarisch and Richter (5), injecting veratrine into the arteries leading to the head in dogs and cats, concluded that the decrease in heart rate was caused essentially by a reflex action, the afferent impulses originating in the heart   Confirmatory evidence of this view was supplied by Richter and Thoma (6), who found in cats that injection of veratrine into the vertebral artery or into the fourth ventricle of the central nervous system only in rare cases caused a decrease in heart rate   This heart rate decrease they ascribed to a reflex action brought about by that part of the veratrine which reached the heart after passage through the circulatory system of the head

It seemed likely to us, from the evidence presented, that there was no real discrepancy in these reports and that the heart rate lowering effect of the veratrum alkaloids might be due partly to reflex action and partly to central vagal action. In order to verify this assumption, 14 experiments were conducted in dogs. Four were done in heart-lung-head preparations in which the circulatory system was reduced to involve only heart, lungs, head, and right foreleg. This preparation excludes to a large extent changes in arterial resistance by substituting the artificial arterial resistance of Starling's heart-lung preparation for the major part of the physiological arterial resistance of the experimental animal. For a more exact analysis of the site of action of the alkaloids, 10 cross-circulation experiments were made in which there was nothing but nervous connections between the central nervous system and the head circulation on one side, and the heart, which was working in a heart-lung preparation, on the other side.

Veratrine hydrochloride Merck was used for one heart-lung-head experiment and for two cross-circulation experiments. In all other experiments veratridine was used; in four of these cevine was tested in comparison with veratridine, and in three veratric acid was also administered. Unless otherwise specified, all dosages mentioned below refer to veratrine hydrochloride, cevine hydrochloride + $2H_2O$ (mol. wt. 581), and veratridine base (mol. wt. 673), respectively.

The veratridine, cevine, and veratric acid used in this study were prepared by Linstead and Todd (7) from the same batch of veratrine hydrochloride Merck that was used in the veratrine experiments of the present investigation and which had been previously studied by Krayer and Mendez (8)

METHOD. The dogs used for the heart-lung-head preparation weighed between 9.0 and 10.2 kgm. They were anesthetized with 0.09 gram of chloralose per kgm. body weight. The animals were heparinized[4] and the preparation was made in a way similar to the heart-lung preparation of Starling, except that after the left subclavian artery and the azygos vein were tied, the cannula for the venous blood supply was introduced into the vena cava inferior and the arterial cannula was placed into the arch of the aorta. The total output of the left ventricle therefore separated into coronary blood, blood flowing through the brachiocephalic artery into the head region, and blood flowing through the aortic cannula into the heart-lung system and returning through the vena cava inferior to the right heart. Only the last fraction was measured by the Weese stromuhr in the system. Defibrinated blood from a bleeder dog was usually used to supplement the total blood volume in the preparation.

The dogs used for the cross-circulation experiments weighed between 8.4 and 17 kgm. They were anesthetized with 0.045 gram of chloralose per kgm. body weight, ether being used to deepen the anesthesia when necessary during the operation. The animals were heparinized, and heparinized blood was obtained from one or two bleeders, so that from 1.2 to 1.8 liters of blood were available for one experiment.

The method of cross-circulation was that described in detail by Krayer and Verney (9). In two of the ten experiments the head was perfused from a second dog by connecting its abdominal aorta with the cranial end of the brachiocephalic artery and allowing the blood to return from the vena cava superior of the perfused head into the abdominal part of the vena cava inferior of the eviscerated perfusing dog. In order to prevent marked changes in arterial pressure and heart rate of the perfusing dog by the injection of the drug into the "head circulation," the vagi of the perfusing dog were severed. In eight experiments a

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[4] The heparin used was obtained from the Connaught Laboratories, Toronto.

pump was used for the perfusion of the head and oxygenation of the blood was achieved by an artificial oxygenator. A compensating device similar to that described by Krayer and Verney made it possible to obviate at will pressure changes in the head circulation.

These cross circulation experiments enabled us to administer the alkaloids into the heart lung circuit or into the head circulation. In five of the experiments the carotid sinus nerves were isolated prior to the beginning of the experiment and were then severed during the experiment in order to evaluate the part played by afferent impulses from the carotid sinus in the reflectoric decrease in heart rate. In isolating the carotid sinus nerves a wide exposure of both carotid sinus areas was obtained by reflecting the larynx and esophagus cephalad (10). All visible vessels were excluded from the nerve ligatures.

The alkaloids and other substances were injected into the venous blood supply of the heart lung preparation or into the arterial blood supply to the head. In the latter case the total amount of substance to be injected was dissolved in not more than 0.2 cc of 0.9% sodium chloride solution. The pH was adjusted to about 7.5 and the solutions were kept at a temperature of 38°C prior to their injection. The alkaloids were administered as hydrochlorides, the veratric acid was given as sodium veratrate.

1 *Experiments on the heart lung head preparation*. It has been shown by Krayer and Mendez (8) that doses of 0.1 to 0.2 mgm of veratrine given into the denervated heart lung preparation to make concentrations of 1.9 million to 1.25 million do not lead to a conspicuous change in the heart rate. If the heart rate is lowered at all, this effect occurs only gradually. It is pronounced only if large doses are repeatedly given, as shown, for example, in table 3 of the paper by Krayer and Mendez, where 0.3 mgm of veratrine, given 3 times in succession, led to a drop in rate from 148 per minute to 88 per minute within a period of 77 minutes. Toxic doses of veratrine given initially were found to cause a slight increase in rate, and irregularities. Similar results were obtained with veratridine and cevine by Moe and Krayer (11). In contradistinction to this, the heart rate lowering effect in the innervated heart is abrupt and marked and is contingent upon the proper functioning of the vagal mechanism, as can be seen from figure 1. Within a few seconds after the administration of veratrine the heart beat ceased for about 10 seconds and then gradually increased to reach the rate of 60 per minute within about one minute (figure 1, A). The heart rate promptly rose when the vagi were cut (fig 1, B), and a subsequent dose was without effect (figure 1, C).

Similar observations could be made with 0.1 to 0.2 mgm of veratridine, while cevine, even in doses of 5 to 10 mgm, did not cause the abrupt lowering of the heart rate. The only effect which could possibly be attributed to cevine was a gradual decrease in rate. In one experiment, for instance, after an injection of 10 mgm of cevine the heart rate fell from 144 to 110 within a period of 13 minutes.

In the experiments with veratridine it was observed that the effect of the first dose wore off more readily than the effect of a subsequent dose and also that the intensity of the initial effect tended to be less with the second dose, even if it was larger than the first. In the experiment of figure 2, for instance, from the return of the heart rate and the absence of any signs of irregularities in the heart beat it is evident that the effect of the initial dose of 0.1 mgm of veratridine, which had caused a decrease to 38% of the original heart rate, had nearly dis

appeared 10 minutes after the injection. However, after the injection of 0.2 mgm. more of veratridine the rate dropped to only 55%, and, after a transient increase, reached 45% of the original rate within 5 minutes. Also, irregularities
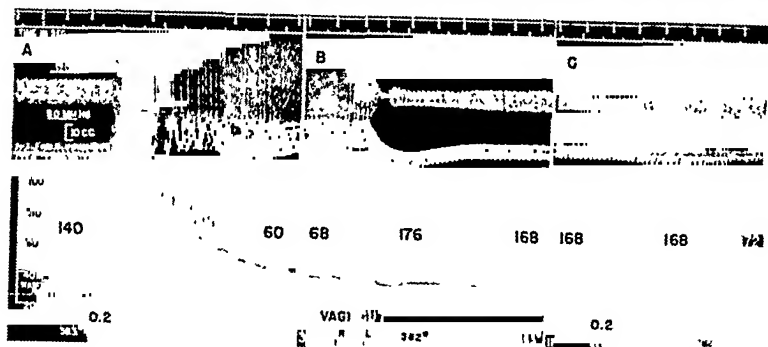
FIG. 1. Experiment March 24, 1941. Action of veratrine upon heart rate before and after cutting the vagus nerves. Heart-lung-head preparation. Dog, 9.5 kgm. 0.09 gram chloralose per kgm. body weight. Weight of heart 56 grams. Arterial resistance 90 mm. mercury. Tracings from top to bottom: systemic output in 100 cc.; time in 10-second intervals; arterial pressure (scale on left in mm. mercury); heart volume (scale on left in cc.); right auricular pressure (scale on left in mm. water). The horizontal rows of figures represent, from top to bottom, heart rate per minute and temperature in degrees centigrade. At the signal in A, 0.2 mgm. of veratrine hydrochloride was injected into the inflow tube. At the signal in B the vagus nerves were cut. At the signal in C, 0.2 mgm. of veratrine hydrochloride was injected into the inflow tube as in A. Between A and B was an interval of 3 minutes; between B and C, 11 minutes.
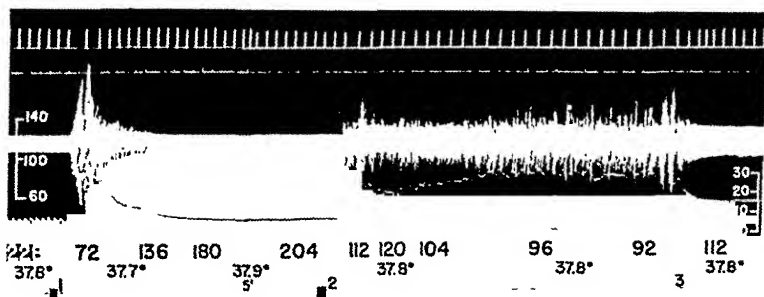
FIG. 2. Experiment November 12, 1942. Action of veratridine upon heart rate. Heart-lung-head preparation. Dog, 9.5 kgm. 0.09 gram chloralose per kgm. body weight. Weight of heart 68 grams. Arterial resistance 78 mm. mercury. Tracings from top to bottom: systemic output in 100 cc.; time in 10-second intervals; arterial pressure (scale on left in mm. mercury); right auricular pressure (scale on right in mm. water). The horizontal rows of figures represent, from top to bottom, heart rate per minute and temperature in degrees centigrade. At 1, 0.1 mgm. of veratridine was injected into the inflow reservoir. At 2, 0.2 mgm. of veratridine was injected in the same way. At 3 the vagus nerves were blocked by cooling. At 5' the drum was arrested for 5 minutes.

of heart rate persisted, indicating a difference in effects. This is probably due to a difference in responsiveness of the preparation, because in another experiment 0.2 mgm. of veratridine, given under comparable conditions as an initial

dose, caused an abrupt decrease in rate to 42% (from 172 to 72) with a subsequent increase to 72% of the original rate within 3 minutes. That these changes are largely, if not entirely, mediated by the vagus can be demonstrated by excluding the vagal impulses. In figure 2 cooling the vagi (at 3) led to a prompt increase in heart rate, and 6 minutes later cutting the vagi caused the heart rate to rise to 128 per minute.

No attempt was made in these heart-lung-head experiments to establish the minimal effective dose of veratridine or to analyze further the mechanism of action.
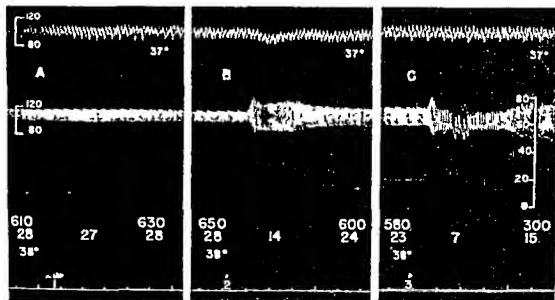
FIG. 3. Experiment February 17, 1942. Action of cevine and of veratridine on nerve impulses in circulation. Heart-lung dog, 9 kgm. Heparin, 100 mgm. Arterial resistance perfused by pump. Tracings from top to bottom: arterial pressure in head circulation (scale on left in mm mercury), arterial pressure in second circulation in cc. temperature in degrees ... into injected C, 9 pensated

2. *Cross circulation experiments*. The use of the cross-circulation technic made it possible to establish the reflex nature of at least a part of the heart rate lowering effect of the veratrum alkaloids. This effect could be obtained with veratrine, as well as with the pure alkaloid veratridine (figure 3). In figure 3, B, 5 micrograms of veratridine injected into the inflow tube of the heart-lung preparation caused the characteristic drop in heart rate, which had worn off largely, but not entirely, 10 minutes later. The initial decrease in rate of 50% was caused by a concentration of approximately 1:40 million to 1:60 million of veratridine in the blood reaching the heart. The dose was repeated in figure 3,

C, and led to a drop in heart rate of 70%. The same dose was subsequently given 3 times more, and the fifth dose still showed a distinct effect, although the heart rate previous to the injection had reached a level of only 96 beats per minute and the decrease to 84 lasted only for about 10 seconds. Cevine was without the characteristic effect of veratrine and veratridine; not even a dose 400 times that of veratridine caused a change in rate, as shown in figure 3, A. Similarly, veratric acid in doses up to 10 mgm. was without effect.

The veratridine response on repetition was considerably weakened if the first dose administered was larger than 10 micrograms under the conditions of our experiments. Occasionally even an initial dose of 10 micrograms led to a weaker
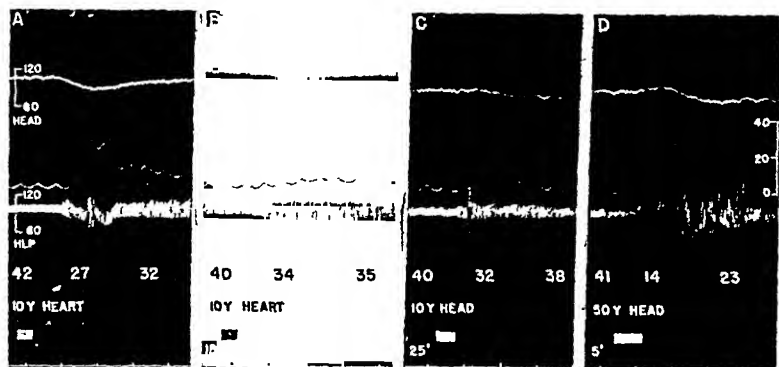
FIG. 4. Experiment December 17, 1941. Action of veratridine upon nerve impulses to heart after injection into the heart and after injection into the head circulation. Cross circulation. Heart-lung dog, 12.5 kgm. 0.045 gram chloralose per kgm. body weight, and ether. Heparin, 100 mgm. Head perfused by pump. No compensation. Blood volume in head circulation 800 cc. Blood volume in heart-lung preparation 500 to 600 cc. Tracings from top to bottom: arterial pressure in head circulation (scale on left in mm. mercury); right auricular pressure of heart-lung preparation (scale on right in mm. water); arterial pressure of heart-lung preparation (scale on left in mm. mercury); signal line; time in 10-second intervals. The horizontal row of figures represents heart rate per 10 seconds. The temperature in the head circulation was 38°C.; in the heart-lung preparation, 39.3 to 39.6°C. At the signal in A and in B, 10 micrograms of veratridine was injected into the inflow tube of the heart-lung preparation. At the signal in C and in D, 10 micrograms and 50 micrograms respectively were injected into the rubber tubing carrying the arterial blood supply to the head. Between A and B was an interval of 18 minutes; between B and C, 25 minutes; and between C and D, 5 minutes.

response, as in figure 4, where the first and second dose caused a maximal drop in heart rate to 64% and 85% respectively of the original rate. The effect in both cases was completely reversible, and the heart returned to the original rate after 18 to 25 minutes.

When in these experiments veratridine was injected into the head circulation, decrease in heart rate was observed with dosages from 10 to 70 micrograms. The effect was proportional to the dose and with a dose of 50 micrograms could be obtained repeatedly (the highest number of injections in one experiment was 5) without the weakening in the response characteristic of large doses given into the heart-lung circulation. In the experiment of figure 4, C, 10 micrograms caused a decrease in rate to 80% of normal, which had disappeared after one minute;

while 50 micrograms lowered the rate to 34%, and after one minute the rate had returned to 61% of the initial rate. After cutting the vagi no response could be obtained with doses of 100 micrograms.

These experiments prove that the heart rate lowering effect cannot be entirely ascribed to impulses originating in the heart lung circuit. As the carotid sinus areas in our cross circulation experiments were included in the head circulation, afferent impulses originating there, as well as impulses originating in the central nervous system, could be responsible for part of the action. In order to evaluate the importance of impulses from the carotid sinus area, injections were made into the head circulation before and after severing the carotid sinus nerves. The
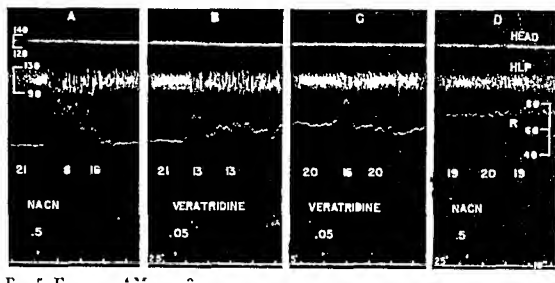
circulation (scale on left in mm mercur...

minutes Head pressure compensated

results of our 5 experiments were not uniform. In three experiments there was a marked weakening of the response as illustrated in figure 5, in one experiment the decrease was slight, and in one experiment no noticeable difference in the effect could be observed after the denervation of the carotid sinus area. In all experiments the completeness of the denervation of the carotid sinus area was verified by the absence of a response to sodium cyanide (chemoreceptors, see fig 5) and to changes in the arterial pressure of the head circulation(pressor receptors) (12)

In the cross circulation experiments the amount of blood in the head circulation was in the range of 800 to 1500 cc, and the circulation rate was between 300 and 450 cc per minute as measured by the Weese stromuhr inserted into the

arterial blood supply to the head. The maximal initial concentration of veratridine entering the head circulation, therefore, if a dose of 50 micrograms was injected within about two seconds, was of the order of 1:200,000 to 1:300,000; and the limit effective concentration, corresponding to a dose of 10 micrograms, was of the order of 1:1 million to 1:1.5 million.

Cevine and veratric acid given into the head circulation were without effect in doses of 2 mgm., corresponding to a maximal initial concentration of 1:5,000 to 1:7,500.

DISCUSSION. The apparent discrepancy in the literature in regard to the central versus reflex nature of the heart rate decrease has been resolved by our experiments. Afferent impulses from the heart-lung circuit, as well as impulses from the central nervous system, take part in the effect upon the intact circulatory system. In addition, our experiments make it probable that afferent impulses originating in the carotid sinus area also partake in the action of veratridine under discussion. This latter observation is not in agreement with statements of Jarisch and Richter (5), but their reference is not detailed enough to allow a critical evaluation. With regard to a more specific localization of the origin of the afferent impulses within the heart-lung circuit our experiments furnish no evidence, and we cannot decide what role receptors in the vessels of the lungs, in the ventricular muscle, and in the ascending aorta play. Since Cramer (13) came to the conclusion that pulmonary receptors were responsible for the reflex decrease in heart rate which he observed with a mixture of alkaloids of *Veratrum viride*, it will be necessary to consider this possibility in a further analysis of the action of pure alkaloids. Jarisch and Richter, on the basis of their work on cats, believed pulmonary receptors to be of little significance. They attribute the greatest importance to receptors of the myocardium. Jarisch and Richter also deny that aortic receptors contribute to the reflex decrease in rate, which, in view of our experience with the denervation of the carotid sinus area, cannot be accepted without further proof.

For a study of the reflex action originating in the heart, our experiments emphasize the necessity of working with a dosage near the minimal effective dose if a readily reversible and reproducible effect is desired. While the nature of the weakening of the response with initial doses of more than 10 micrograms under the conditions of our experiments is not fully understood, we assume that such doses influence the receptor mechanism, as the central effect was not weakened, indicating that the efferent pathway of the reflex arch remained unchanged. A similar view was expressed by Cramer (13) in his work on *Veratrum viride*. He states that a marked slowing of the heart, which in cats is sometimes absent, could only be obtained with small doses, and that the drug, after having stimulated the afferent nerve endings of the vagus, paralyzes them so that a second and third dose is without effect.

Mendez and Montes (14) have recently emphasized some qualitative differences between veratridine and cevine. (The ester-alkaloid veratridine can be split into the alkamine cevine and veratric acid by hydrolysis in alcoholic potassium hydroxide solution.) Our experiments reveal another difference between

these two substances in that the reflectoric as well as the central action involved in the lowering of the heart rate can be obtained only by the ester-alkaloid veratridine, and not by cevine. Veratric acid also was found inactive. The specificity of the action of the ester-alkaloids of cevine does not seem to depend upon the nature of the organic acid, as Lissauer (2) and Pilcher and Sollmann (3) in intact animals observed effects with cevadine, the tiglic acid ester of cevine, which are similar to those we saw with veratridine.

Whether or not there is a true qualitative pharmacological difference between the alkamine cevine and its ester-alkaloid veratridine is difficult to ascertain as long as the biochemical mechanism by which the ester-alkaloid acts remains unknown. It is conceivable that cevine is the active compound even in the administration of veratridine, and that the ester-alkaloid is instrumental only in the distribution of the alkamine, enabling it to reach the site of its specific action.

SUMMARY

In cross-circulation experiments in which the heart-lung system was in connection with the central nervous system by nervous pathways only, the veratrum alkaloid veratridine, when administered into the heart-lung circulation, caused a decrease in heart rate in a dose too small to have a direct action upon the impulse generation in the heart. This effect of veratridine was found to be mediated by the vagus, the afferent impulses originating within the heart-lung circuit. A heart rate decrease could also be obtained when the alkaloid was injected into the circulatory system of the head. The available evidence indicates that afferent impulses responsible for the reflex decrease do not arise in the heart or lungs alone, but also in the carotid sinus area, and that there is in addition to the reflex effect a direct effect upon the central nervous system causing vagal stimulation.

For a study of the reflex response originating in the heart-lung circulation it was found imperative to work with dosages close to the minimal effective dose, as large initial doses diminished or abolished a subsequent response. With the dosages used, such a weakening was not observed with regard to the central effect.

The reflectoric as well as the central heart rate lowering effect were caused by the ester-alkaloid veratridine but not by either of the two products of its hydrolysis, cevine and veratric acid.

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THE SKIN ABSORPTION OF TRIORTHOCRESYL PHOSPHATE AS SHOWN BY RADIOACTIVE PHOSPHORUS[1]

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Triorthocresyl phosphate became of interest toxicologically in 1930 when the ginger paralysis (or ginger jake) was traced to the presence of triorthocresyl phosphate as an adulterant in certain fluid extracts of Jamaica ginger   Some interesting pharmacological properties of triorthocresyl phosphate were subsequently described (1, 2, 3) for example, 1) the latent period before paralytic symptoms develop, 2) the widely differing susceptibility of different species of animals, 3) the selective affinity of this compound for nerve tissues, and 4) the cumulative action of relatively minute doses over a prolonged time interval When recent technical developments indicated that triorthocresyl phosphate may have wider industrial applications, the problem of the routine handling of the material arose, and with it the question of toxic effects from chronic absorption through the skin   Since skin absorption is presumably not of a large magnitude, the use of a radioactive tracer appeared to be singularly useful in attempting to answer the questions—How much triorthocresyl phosphate can be absorbed through the unbroken skin and how rapidly does this absorption occur?   This problem has been attacked using triorthocresyl phosphate containing radioactive phosphorus, and the absorption has been measured in 2 human subjects and in a dog

PREPARATION OF THE TRIORTHOCRESYL PHOSPHATE WITH RADIOACTIVE PHOSPHORUS   The triorthocresyl phosphate containing radioactive phosphorus was prepared by Dr A Bell of the Synthetic Organic Research Laboratory of the Eastman Kodak Company   A sample of red phosphorus was bombarded by deuterons in the cyclotron of the University Physics Department   This sample was diluted with inert red phosphorus and treated with sulfuryl chloride to make phosphorus trichloride which was converted to phosphorus oxychloride using potassium chlorate   The phosphorus oxychloride was distilled and added to sodium cresylate in benzene   The product in benzene was washed with dilute alkali, acid, bi carbonate and water   The benzene was removed by distillation under reduced pressure on the steam bath   The resulting triorthocresyl phosphate (slightly colored) represented a yield of approximately 30 per cent

Skin absorption in humans   Two subjects applied weighed amounts of triorthocresyl phosphate containing marked phosphorus to the palms of both hands and rubbed the material briskly until it was evenly distributed   Subject S applied 0 22 g , subject H 0 11 g at 8 30 a m   This sample of triorthocresyl phosphate had a radioactivity of 395,000 counts per minute per 0 1 g , these

225

counts were made on the scale of four Geiger-Muller counter (4). The triortho-cresyl phosphate was removed at 12 noon of the same day (3½ hours exposure) by washing the hands in appropriate solvents. Blood and urine samples were collected at the hours shown in tables 1 and 2. The rapidity with which triortho-cresyl phosphate entered the blood stream was striking; subject S had 13 micrograms of triorthocresyl phosphate per 100 cc. of blood at the end of one hour, subject H had 4 (table 1). These high levels were not maintained perhaps due to urinary excretion (table 2) or perhaps due to a fixation in the tissues. Thus, although detectable amounts were still present in the blood 24 hours later, no radioactivity was found after 48 hours. The urinary excretion of triorthocresyl phosphate began promptly; subject S excreted 7 micrograms in the first hour after application, subject H excreted 10. In subject S, the urinary excretion rate rose to a level of 35 to 40 micrograms per hour which rate continued for nearly 24 hours. In subject H, no such high values were found, instead the rate varied between 2 and 9 micrograms per hour during the first 24 hour period. Forty-eight hours following the application, triorthocresyl phosphate was still being excreted at rates of 8 and 2 micrograms per hour in subjects S and H respectively. The excretion rate had fallen to 2 micrograms per hour in subject S 72 hours after the application. Considering the small absorbing area, the total amounts of triorthocresyl phosphate excreted via the urine were surprisingly large. Subject S excreted 797 micrograms of triorthocresyl phosphate, or 0.36 per cent of the amount applied; subject H excreted 143 micrograms, or 0.13 per cent of the amount applied.

Both the amounts of triorthocresyl phosphate absorbed through the palmar surfaces and the duration of the urinary excretion following a few hours contact are worthy of note. Smith, Engel and Stohlman (1) have shown that in chickens, triorthocresyl phosphate has a marked cumulative action. They found that "the daily ingestion of very small, and in themselves ineffective doses, of triortho-cresyl phosphate over a period of days is certain to give rise to flaccid paralysis." There is no way of knowing whether daily exposures of the order tested in this experiment would by cumulative action result in the syndrome of "ginger paralysis" in man. However, the danger from skin absorption is plain and precautions should be taken to protect workmen who handle triorthocresyl phosphate routinely.

Skin absorption in a dog. A suitable area on the abdominal surface of a 10.5 kg. female dog was prepared on the day preceding the experiment by clipping the hair and then applying a depilatory. At 8:45 a.m., March 5, 1941, an area roughly rectangular, 15 x 20 cm., and located with about ⅓ of the area anterior to and ⅔ posterior to the xiphoid was coated with triorthocresyl phosphate containing radioactive phosphorus. The triorthocresyl phosphate was applied with a camel's hair brush and was not rubbed in. The triorthocresyl phosphate was rinsed from its container with a small portion of the same material (not radioactive). The total amount of triorthocresyl phosphate applied was 2.094 g. which contained 5,575,000 counts per minute of radioactive phosphorus. Since it was necessary to keep the dog quiet, nembutal in solution was administered

from time to time as needed Thirty-five hundred cubic centimeters of a 2½ per cent dextrose in Ringer's solution was given hypodermically at 11 45 a m

TABLE 1

Blood levels of triorthocresyl phosphate in humans following its application to the palms of the hands

DATE	HOUR	RADIOACTIVITY COUNTS PER MIN PER 10 CC BLOOD SAMPLE		% DOSE PER 100 CC BLOOD		MICROGRAMS T C P PER 100 CC BLOOD	
		Subject S*	Subject H†	Subject S	Subject H	Subject S	Subject H
		cpm	*cpm*	$\times 10^2$	$\times 10^2$	γ	γ
3/5/41	8 30 a m	T C P	applied	to palms	of hands		
	9 30 a m	1 1	0 7	0 52	0 40	13	4
	11 45 a m	0 4	0	0 22		4	
	3 45 p m	0 2	0 3	0 01	0 18	2	2
	8 30 p m	0	0				
3/6/41	9 15 a m	1 4	0 5	1 1	0 68	21	7
3/7/41	9 00 a m		0				

* Subject S applied 0 22 g of triorthocresyl phosphate to palms
† Subject H applied 0 11 g of triorthocresyl phosphate to palms

TABLE 2

Urinary excretion of triorthocresyl phosphate in humans following its application to the palms of the hands

DATE	COLLECTION HOURS	RADIOACTIVITY COUNTS PER MIN PER WHOLE URINE SAMPLE		MICROGRAMS T C P EXCRETED		RATE OF EXCRETION OF T C P	
		Subject S	Subject H	Subject S	Subject H	Subject S	Subject H
		cpm	*cpm*	γ *gm*	γ *gm*	γ/hr	γ/hr
3/5/41	8 30 a m	T C P	applied	to palms	of hands		
	8 30–9 30 a m	29	38	7	10	7	10
	9 30–11 45 a m	115	56	37	14	16	7
	11 45 a m – 3 45 p m	587	66	148	17	37	4
	3 45– 8 30 p m	327	40	83	10	17	2
	8 30 p m –12 m	477	162	120	41	34	9
3/6/41	12 m –7 00 a m	1180	157	299	40	43	8
	7 00– 8 30 a m	47		12		8	
3/7/41	9 30 p m – 6 30 a m	282		71		8	
	2 00– 7 00 a m		43		11		2
3/8/41	10 30 p m – 8 30 a m	76		19		2	

and 500 cc intraperitoneally at 10 30 p m Blood samples (25 cc) were collected at intervals during the experiment, bladder urine samples were obtained

by catheterization for the periods between blood samples. The dog was sacrificed by exsanguination at 10:00 a.m., March 6, 1941 ($25\frac{1}{4}$ hours after application), and the blood and various tissue samples taken. Some of the tissue samples were ashed with fuming nitric acid, others were dissolved in a 10 per cent NaOH solution. The samples were counted on a Geiger-Muller counter, and, from simultaneous counts on a standard aliquot of the triorthocresyl phosphate, all counts were calculated to a zero time point for comparison.

The blood level (table 3) of triorthocresyl phosphate established itself with extraordinary promptness at about 8 micrograms per 100 cc. by the end of the first hour and maintained itself at this level during the ensuing 24 hours. The values varied only from 5 to 10 micrograms per 100 cc. (average 8 micrograms per 100 cc.) of blood and are of approximately the same order of magnitude as the triorthocresyl phosphate levels observed in the human tests in which all the values except one fell between 2 and 13 micrograms per 100 cc. (average—8 micrograms per 100 cc.).

TABLE 3

Blood levels of triorthocresyl phosphate in a dog following its application to the abdominal surface

DATE	HOUR	RADIOACTIVITY COUNTS PER MIN. PER 25 CC. BLOOD SAMPLE	% DOSE OF T.C.P. PER 100 CC. BLOOD	MICROGRAMS T C.P. PER 100 CC. BLOOD
		cpm.	$\% \times 10^1$	γ
3/5/41	8:45 a.m.	T.C.P. applied to surface of abdomen		
	9:45 a.m.	21	0.38	8
	10:45 a.m.	14	0.25	5
	3:30 p.m.	25	0.46	10
	9:30 p.m.	0		
3/6/41	10:00 a.m.	24	0.44	9

The urinary excretion of triorthocresyl phosphate in the dog began equally promptly; in the first hour after the application, 44 micrograms were excreted (table 4). The excretion rose to a maximum of 1312 micrograms during the 3:30 to 10:00 p.m. ($6\frac{3}{4}$ to $13\frac{1}{4}$ hours after application) period and fell somewhat to 1097 micrograms during the succeeding 11 hours. An increase in the urinary excretion of phenols, free and total, has been observed in cats (1). The urinary excretion rate, initially about 35 micrograms per hour, doubled during the second hour, trebled during the succeeding 6 hours, increased to six-fold in the next 6 hour period and finally decreased to a three-fold level by the end of 24 hours. The total triorthocresyl phosphate excreted was 2953 micrograms or 0.14 per cent of the amount applied. This is almost exactly the dose percentage excreted by subject H. in the human test and only one-third of the percentage excreted by subject S.

Only a rough comparison of the absorption of triorthocresyl phosphate through human palmar skin and skin from the abdominal area of the dog is permissible

from the data However, it is interesting to note that although the duration of
contact in the human experiment was only about 15 per cent that of the dog experiment, and the human area treated only about $\frac{2}{3}$ that of the application area of
the dog, the rapidity of transfer through human palmar skin appears to be about
ten times faster than through the abdominal dog skin This difference is further
accentuated if, in addition, the amounts applied are considered (dog, 2 1 g ,
human, S—0 2, H—0 1 g), with human palmar skin apparently transferring
triorthocresyl phosphate about 100 times more rapidly than does dog skin

The distribution of triorthocresyl phosphate in the tissues (table 5) shows that
certain tissues have special abilities to fix this substance As would be expected,
the skin of the area treated, from the surface of which the applied triorthocresyl
phosphate was removed by repeated and prolonged washings with alcohol before
excision, was found to contain relatively large amounts of radioactive material
(73 micrograms per g of skin) The underlying fascia and superficial muscles
contained only 3 micrograms per gram, and the omental fat 2 micrograms per

TABLE 4

Urinary excretion of triorthocresyl phosphate in a dog following its application to the abdominal surface

DATE	COLLECTION HOURS	RADIOACTIVITY		TCP EXCRETED	RATE OF EXCRETION OF T C P
		Counts/m n per whole urine sample	% dose per whole urine sample		
		cpm	$\times 10^3$	γ	γ/hr
3/5/41	8 45–10 00 a m	118	0 21	44	35
	10 00–10 50 a.m	166	0 30	62	75
	10 50 a m – 3 30 p m	1155	2 1	437	94
	3 30–10 00 p m	3495	6 3	1312	202
3/6/41	10 00 p m – 9 00 a m	2924	5 2	1097	100

gram The only tissue with more triorthocresyl phosphate than these was the
liver with 5 micrograms per gram This evidence of the role of the liver in
handling and perhaps detoxifying triorthocresyl phosphate, is in accord with the
finding of Lillie and Smith (2) that "fine droplet fatty degeneration" occurred in
centrolobular areas in certain cats with various degrees of chronic poisoning
The other viscera examined had 0 3 to 0 9 micrograms of triorthocresyl phosphate
per gram Muscle contained 0 49 micrograms per gram and femur 0 18 micrograms per gram Because triorthocresyl phosphate has been shown from human
symptomatology and from histological studies on cats (2) to have marked
neurotoxic actions, it was of special interest to examine the brain, spinal cord and
one of the peripheral nerves, the sciatic The brain contained 0 2 micrograms per
gram, the spinal cord—0 3 micrograms per gram, and the sciatic nerve 0 2 micrograms per gram of triorthocresyl phosphate These figures are of the same
magnitude as those for heart, spleen and muscle and somewhat higher than that
for the femur

The indication from this comparison that the nerve tissues have unusual affinity for triorthocresyl phosphate is not self-evident but it becomes plainer if the distribution of disodium acid phosphate into the same tissues is used as a basis.   In rats (5, 6), mice (6, 7), and rabbits (6), it has been shown that this inorganic phosphate regardless of the route of administration, appears in largest amount in bone.   The viscera, such as liver, heart, lung, and kidney, usually contain only one half, and frequently much less, as much marked phosphorus as the bone.   Muscle and brain are usually lowest and may have one-third or less of the marked phosphorus content of the visceral organs, gram for gram.   Thus, in the distribution of inorganic phosphate, the tissues arranged in order of per-

TABLE 5

Distribution of triorthocresyl phosphate in the tissues of the dog 24 hours following its application to the abdominal surface

TISSUE	WEIGHT SAMPLE	RADIOACTIVITY		T C P PER G TISSUE
		Counts per min in entire sample	% dose per g tissue	
	g	*cpm*	$\times 10^{-4}$	γ
Skin*	33 0	8775	0 47	73
Fascia†	47 0	497	0.019	3
Fat‡	47 0	393	0 015	2
Liver	340 3	5720	0 030	5
Blood	300 cc	1487	0 009	1
Kidney	55 4	173	0 006	0 9
Heart	81 0	95	0.002	0.3
Lung	116 8	282	0 004	0.7
Spleen	22 8	14	0.001	0 2
Muscle§	224 0	317	0 002	0 4
Femur	50 0	17	0 0006	0.1
Brain	69 4	39	0 001	0.2
Spinal cord	5 2	5	0 002	0 3
Sciatic nerve	10 0	6	0 001	0 2

* Abdominal skin from the site of the triorthocresyl phosphate application
† Fascia and superficial muscles underlying the area of application
‡ Omental fat
§ A sample of the triceps femoris

centage of the dose picked up per gram of tissue are as follows: visceral organs, bone, muscle, brain.   In the triorthocresyl phosphate distribution, a similar comparison of the tissues is as follows: visceral organs, muscle, brain, bone. Since inorganic phosphate is found only in traces in bone, the assumption seems valid that little or no hydrolysis of the triorthocresyl phosphate occurred either before or after absorption.   This is important since on this experimental evidence it can be argued that the marked phosphorus found in various tissues and in the urine was triorthocresyl phosphate, or at least, not an inorganic degradation product
    To return to the question of the affinity of triorthocresyl phosphate for nerve

tissue, it may be seen from table 5 that the brain contained 0 2 micrograms per gram, the spinal cord—0 3, and the sciatic nerve—0 2   This is as much or more than that found in the heart—0 2 micrograms per gram   24 hours after a dose of inorganic phosphates (5), the heart has been known to contain about twice as much marked phosphorus as the brain   Thus, the selection by the brain and other nerve tissue of triorthocresyl phosphate appears to be greater than occurs with the inorganic phosphates   This is in line with the predominately neurotoxic action of triorthocresyl phosphate

Acute toxicity in mice   Data (1) on the acute toxicity of triorthocresyl phosphate for rabbits, guinea pigs, chickens, cats, and dogs are available   The rat has been found to be atypical and highly resistant to the paralysis caused by this compound   To check the latter observation, six female 90 g rats were given intraperitoneally a dose (5 cc per kg) of triorthocresyl phosphate (larger than the fatal dose in mice) and no toxic effects were noted   The possibility of the use of mice as experimental animals led to the determination of the mean lethal dose (L D 50) in mice

Ninety six young albino mice, average weight about 20 g , were given graded doses of triorthocresyl phosphate intraperitoneally   Groups of 24 mice were treated as shown in table 6   The number dead on each of the first four days is indicated   The L D 50 was found by the method of Bliss (8) to be 0 056 cc for the average mouse or 2 8 cc per kg   Two significant points are found 1) triorthocresyl phosphate is only $\frac{1}{5}$ to $\frac{1}{15}$ as toxic for mice as for cats, dogs, rabbits etc , and 2) the lethal effect does not appear promptly   Almost $\frac{1}{3}$ of the mice killed by 0 07 cc died on the third day and 5 of the 21 killed died on the 4th day   This delay is in line with the similar action on other animals and was more or less expected   A few mice exhibited the flaccid paralysis of the hind legs frequently described in other animals   Two such mice are shown in figure 1   The absence of a regularly occurring paralysis in mice made the use of this species in skin absorption studies impractical

DISCUSSION   The above studies were made following a proposal that triorthocresyl phosphate be used in an industrial operation which if carried on in the usual manner, would involve a frequent and considerable skin contact by the operators   The following information, obtained from the literature on the subject indicated that there might be a real hazard involved, but no definite data were available as to the magnitude of absorption through the human skin,—a crucial factor in evaluating the hazard

1) The amount of triorthocresyl phosphate which will cause poisoning although not definitely defined, is apparently relatively small   Smith (3) states, "judging from the chicken experiments, as little as 2 grams of the poison and possibly less might have been sufficient to cause a moderate degree of paralysis in man "   Von R Staehelin (9) in reporting a series of cases of paralysis following the ingestion of a cheese cake contaminated with triorthocresyl phosphate estimated that as little as 0 15 grams produced poisoning, 0 5 to 0 7 grams severe poisoning

2) The cumulative action of triorthocresyl phosphate as demonstrated in the

chicken is striking.   Smith et al. (1) report, "the daily ingestion of very small, and in themselves ineffective doses, of triorthocresyl phosphate over a period of days is certain to give rise to flaccid paralysis of the extremities, provided that the total dose ingested approaches its minimum paralyzing dose."

3) Absorption of triorthocresyl phosphate through the ear canal skin of the rabbit, sufficient to cause death after 4 to 17 days was demonstrated by Gross and Grosse (10).

TABLE 6

Acute toxicity of triorthocresyl phosphate in mice—intraperitoneal injection

NO. OF MICE	DOSE	NO DEAD				% MORTALITY
		Day 1	Day 2	Day 3	Day 4	
	cc.					
24	0.01	0	0	0	0	0
24	0.05	0	3	1	3	29
24	0.07	2	7	7	5	87
24	0.10	0	20	3	1	100
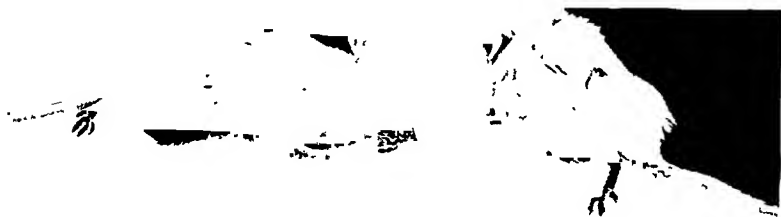
FIG. 1. TWO MICE GIVEN 0.07 CC. OF TRIORTHOCRESYL PHOSPHATE

The weakness in the hind legs is apparent.   In the mouse on the left the front legs were also weakened to the point where the body weight was supported only with difficulty.

4) Triorthocresyl phosphate is not an active primary skin irritant, nor does it produce cutaneous sensitization, so that workmen handling the material would judge it to be innocuous.

5) The character of the syndrome produced by the absorption of triorthocresyl phosphate is such that the "subclinical" or marginal zone of the disease is extremely difficult to diagnose and evaluate.   This factor lessens the acuity of the medical examination in the industrial hygiene control program.   In addition, there is no laboratory examination which will indicate absorption of small

amounts of triorthocresyl phosphate, or early reversible functional changes due to its absorption

The demonstration in these studies of an appreciable absorption of triortho cresyl phosphate through human palmar skin (as shown by an excretion on one subject of 0 36 per cent following exposure to only 0 2 cc for 3½ hours) emphasizes the need for preventing skin contact in occupational situations requiring a considerable or repeated exposure to that compound   In the instance which initiated these studies as originally proposed, an industrial operation necessitating repeated skin contacts with triorthocresyl phosphate, it was recognized that a real hazard existed, and the operating procedures were finally changed so as to eliminate this dangerous factor   Since the compound does not irritate the skin and has such an insidious effect after absorption, workmen exposed to triortho cresyl phosphate must be adequately instructed as to the hazard involved   The supervision must be convinced as to the serious nature of the problem, and must constantly enforce the regulations set up to prevent skin contamination if a safe and healthful working environment is to insured   The use of the radioactive isotope permitted the demonstration of absorption of triorthocresyl phosphate through the human and dog skin with an acuity, ease, and irrefutability not possible by other methods

CONCLUSIONS

1 Triorthocresyl phosphate containing radioactive phosphorus was absorbed through the palmar skin of human hands and through the abdominal skin of a dog

2 Blood levels of triorthocresyl phosphate (average 8 micrograms per 100 cc of blood) were found in the 24 hours following the skin application

3 Urinary excretion of triorthocresyl phosphate amounted to 0 1 and 0 4 per cent of the dose applied to human skin in 2 cases, and 0 1 per cent in the dog test

4 Triorthocresyl phosphate was distributed in the various tissues of the dog, the retentions were in the following order, visceral organs, muscle, brain, bone

5 In an acute toxicity test on mice, the dose required to kill the average mouse was of the order of 0 28 cc per kilogram body weight

6 The magnitude of absorption of triorthocresyl phosphate through human skin is such that a real hazard exists in industrial operations permitting a con siderable or repeated exposure to this compound   A safe industrial hygiene control requires that measures be taken to prevent such skin contact, and that all workmen exposed to the compound be instructed as to the hazard, and the necessity for preventing skin contamination

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EXPERIMENTS ON THE PHARMACOLOGY OF RESPIRATION IN THE RABBIT

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Respiratory stimulants often produce both an increase in depth and rate of respiration, while depressants produce in some cases the reverse   The purpose of these experiments was to ascertain how such effects were produced and the site of action of the various drugs   The effect of any drug on the respiration might be modified by its action peripherally or on higher portions of the nervous system   Consequently an endeavour was made to separate reflex effects from central ones

THE VAGO PULMONARY REFLEX   The technique employed was essentially that of Ferguson (1)   The rabbit was under light urethane or chloralose anaesthesia A difference between these anaesthetics could not be recognized   The effect of inflation or deflation was recorded with a gasometer recorder   Change in respiratory rate was obtained by measuring under a hand lever with a transparent scale graduated in 0 5 mm two respiratory cycles prior to and after the pressure change was made   The change was expressed in % of the preceeding rate Also an attempt was made to match the change produced by any drug by varying the inflation or deflation pressure   For example, in one experiment a plus or minus pressure change of 1 cm water produced percentage changes almost identical with 3 cm after nikethamide (coramine) had been given

Normally the respiratory rate is increased by deflation and decreased by inflation   A characteristic result with the same pressure change of ±3 cm , were before cocaine −19 and +48%, after −8% and +11%, before morphine −15 and +23%, after −33% and +98%

All drugs were tested as the initial drug administered in at least two experiments in doses known to produce a marked change in respiration, but in any experiment depressants and stimulants were frequently given alternately and doses had to be varied in view of the state of the animal   However, the results on these reflexes were the same and all tests may be considered together   Cocaine, metrazol, ether nikethamide, picrotoxin, strychnine, nicotine and cyanide increased respiratory rate and ventilation and decreased both reflexes in all cases, with the exceptions noted below , while morphine, barbitone and chloral made these reflexes more effective   In some cases the changes produced on the reflexes were too slight to be measured, these are not considered

The exceptions are illuminating   Nicotine 0 01 mgm /kg produced such a short lived increase in rate that an effect could not be shown   With 0 025 mgm /kg the result was the normal one reported above   Larger doses, over 0 06 mgm /kg , produced a decrease in respiration and then the reflexes were more effective instead of less so   So, too, cyanide in doses about 1 3 mgm /kg

decreased respiration and made the reflexes more effective. In one case with cocaine where rate of respiration was decreased, again the reflex became more effective.

It seemed that when the effect of a drug was to make the respiratory cells discharge more frequently, either due to effects on the chemoreceptors (smaller doses of nicotine or cyanide) or on the respiratory cells themselves, sensory impulses from the lungs had to be increased in intensity to produce their normal effects.

In all the subsequent experiments the animal was enclosed in a body plethysmograph and respiration recorded with a gasometer recorder.

CHEMORECEPTOR REFLEXES. As it was found impossible to be sure of the sinus nerve, and ligating out the region as done by Zunz and Tremonti (2) seemed inadvisable, the carotid was denervated by the section of the glossopharyngeal at its entrance to the skull. Vagus and depressor were both cut to remove reflexes from the aortic body. Effective doses of the stimulants whose action did not persist long, could be given before and after denervation, which was often done in two stages, glossopharyngeal before vago-depressor. Nicotine 0.05 mgm. and cyanide 0.1 mgm. per kg., which increased respiratory rate some 10–20% and ventilation 20–30%, had approximately the same effect when the glossopharyngeal was cut, but none when the denervation was completed. Their effect was then on the chemoreceptors. Nicotine 0.15–0.25 mgm./kg. increased frequency some 10% or more after denervation, but depth was in some cases decreased. With cocaine in doses up to 2.5 mgm./kg., there was usually (4 out of 6 case) a greater increase in rate and total ventilation before than after denervation. But no dose could be found which acted merely on the chemoreceptors.

Nikethamide 5 mgm., metrazol 10 mgm., strychnine 0.5 mgm., caffeine 5 mgm. and picrotoxin 1–2 mgm. seemed to produce the same increase in frequency before and after removal of the chemoreceptors, but the increase in total ventilation was somewhat less. These doses decrease the blood carbon dioxide slightly and this may offset any slight effect of the drugs on the reflex mechanisms.

The dose of morphine required to produce respiratory depression varied greatly from animal to animal and 3 mgm. to 12 mgm. were used. In some cases cutting the chemoreceptor afferents led to a temporary respiratory arrest if the effect of morphine had been marked. In one animal 3 mgm. of morphine decreased respiratory frequency to an irregular 10 per minute. Giving pure oxygen had no effect, cutting the glossopharyngeal led to a decrease to 8 and of the vagodepressors to 6; after which respiration increased slowly to 9 with an even greater total ventilation than before denervation. Though it was evident that the anoxia secondary to the decreased ventilation offset in some cases and to a certain extent the depression produced by morphine, the main effect was on the respiratory cells.

Barbitone, 150–250 mgm., decreased respiratory rate 15–20%; giving pure oxygen again decreased the rate by some 8% and volume 10%. Cutting the

glossopharyngeals and depressor decreased the rate 30%, but increased the total ventilation in two cases.

Both morphine and barbitone decrease the rate of respiration in an animal with the chemoreceptors and vagus cut, but owing to their prolonged period of action quantitative comparisons could not be made.

Ether increased the rate slightly, 2–3%, or not at all, after cutting the chemoreceptor afferents much less than before denervation.

Carbon dioxide in 2% and 5% concentrations in a mixture containing at least 21% of oxygen, increased the respiratory rate by 20% or more, but after cutting the chemoreceptor afferents the increase in rate was less. For example in one case 5% CO_2 the normal increase was 50%, after cutting the glossopharyngeal 40% and after cutting the vagodepressors only 11%. In no case did 2% CO_2 increase the rate more than a doubtful 1% (6 cases). In 6 cases with the afferents cut, 5% CO_2 increased the rate only 2% or 3% or not at all, 10% then produced an increase in rate of 10–15%. The depth was in all cases greatly increased.

CEREBRAL EFFECTS. The above-mentioned drugs were tested before and after decerebration by a cut through the brain stem above the red nucleus so as to avoid tonus changes. Their effect seemed to be quantitatively and qualitatively the same as those in the animal under light urethane anaesthesia.

As Loewy has shown that carbon dioxide as a respiratory stimulant was less effective after morphine, its effect on the respiratory cells might be regarded as that of decreasing the sensitivity of the centre to carbon dioxide. Consequently a considerable number of arterial blood CO_2 analyses were carried out. Morphine in the small doses used and dependent upon the change in total ventilation, increased the blood CO_2 from 2–7%. The respiratory stimulants, nikethamide, metrazol and nicotine, all decreased the blood CO_2 and again there was a rough parallelism between the fall of CO_2 and ventilation. Cocaine, however, was an exception in that while it might increase rate greatly, the depth was decreased and in many cases while total ventilation was increased, alveolar ventilation obviously decreased as the blood CO_2 rose, often 1–3%. As estimates had been made of the dead space in rabbits which showed that it varied from 4–5 cc., largely dependent on the bore of the trachea, calculations of alveolar ventilation from the tracings again showed a decrease in alveolar ventilation in these cases.

Further, if animals were given 2% of CO_2, 21–23% oxygen and nitrogen to inhale, blood CO_2 often rose but slightly—out of 12 experiments less than 1 cc. in 4; less than 2 cc. in 6; less than 3 cc. in 8; over 3 cc. in 1; over 4 cc. in 2; and over 6 cc. in 1. The normal blood carbon dioxide varied from 35–60 cc., mostly about 45 cc. Yet even in those cases where compensation was most perfect and the respiratory cells were highly active, a decrease in blood CO_2 was produced by nicotine 0.1 mgm., metrazol 10 mgm. and nikethamide 5 mgm. Cocaine in the fourth led to a rise in CO_2 of 1.3%. Certainly the increase in rate with cocaine does not seem to depend on increased sensitivity of the centres to CO_2.

As further we have found that no increase in rate of respiration was produced

by 2% CO_2 when the chemoreceptor afferents and vagus are cut, while great increases, 60–70% or more, could be produced by such doses of the respiratory stimulants as mentioned in the preceding paragraph, it seems hardly likely that the effect of the drugs on respiratory cells is due to increased sensitivity to CO_2 alone.

Carbon dioxide blood concentration affects depth more than rate and the increased rate of ventilation produced by the respiratory stimulants does not apparently lead to as great an increase in alveolar respiration as it might do were not the blood level of carbon dioxide reduced.   It is, indeed, characteristic of the tracings that the increase in depth in the first few respirations decreased rapidly, though this may be due to decreasing concentration.

DISCUSSION.   Some of the facts presented are but confirmations of the observations of others, usually in another animal species.   The effect of small doses of cyanide and nicotine on the chemoreceptor has been made by Heymans (7); Comroe and Schmidt (8); Winder, Winder and Gesell (9); and the central effect by the latter.   Marshall and Rosenfeld (10) have shown that the chemoreceptors aid in maintaining respiration after morphine; Beecher and Moyer (11) have shown the same for certain of the barbiturates.

The conclusion that nikethamide does not act on the chemoreceptors differs from the opinion of Zunz and Tremonti (2).

The precise central cells affected cannot be stated with certainty, the nucleus of the tractus solitarius might be involved, but in the denerved animal this seems improbable.   Though Koll (4) has presented evidence that metrazol acts on the anterior horn cells or the adjacent connector cells in the path of the pyramidal tract, such a site can hardly account for an increase in rate.

The base line of the tracings with the same dosage of any drug never seems to rise so abruptly after denervation as before.   This may be due to changes in balance of activity between the expiratory and inspiratory cells, or more probably is due to a change in the tonus mechanism which, as Schoen and Hempel (5) have shown, may occur even during a period of respiratory arrest, and indeed the change shown with nikethamide resembles what these authors describe as drugs producing the increased tonus type.

SUMMARY

1. Evidence is presented in this paper that the stimulant drugs, cocaine, nikethamide, metrazol, nicotine, cyanide, picrotoxin, strychnine and caffeine, in appropriate doses, produce an increase in respiratory rate by acting on the respiratory cells.

2. That the depressant drugs, morphine and barbitone, decrease respiratory rate by acting again on the respiratory cells.

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STUDIES ON SHOCK INDUCED BY HEMORRHAGE

V. The Effect of Cellular Damage on the in Vitro Hydrolysis and Synthesis of Cocarboxylase by Liver Tissue[1]

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INTRODUCTION. It has been found (1) that in dogs subjected to shock induced by hemorrhage, and in animals in anoxic anoxia, tissue cocarboxylase becomes dephosphorylated. The administration of thiamin to shocked animals results in resynthesis of cocarboxylase.

Ochoa (2) has shown that avitaminous pigeon liver, in slices, dispersions, or brei, can synthesize cocarboxylase from thiamin under aerobic conditions. He also found that the preformed tissue cocarboxylase remained constant for several hours if the tissue were incubated in oxygen at 28°, whereas if the atmosphere was changed to one of nitrogen, the cocarboxylase became hydrolyzed, probably by a phosphatase. A number of intermediate reactions may be involved but the final result may be summarized as follows:

$$\text{Cocarboxylase} \underset{O_2}{\overset{N_2}{\rightleftarrows}} \text{thiamin} + \text{phosphate (or thiamin monophosphate)}$$
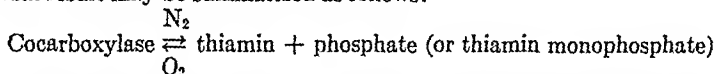
This would seem to indicate a balance between synthesis and hydrolysis of cocarboxylase, aerobic conditions favoring synthesis, and anaerobic conditions increasing hydrolysis.

Since Ochoa's *in vitro* experiments seemed to parallel our *in vivo* work, we repeated his experiments, using however normal rather than avitaminous livers of both pigeons and rats. We were able to obtain results with pigeon liver slices and homogenate which were identical with those of Ochoa. Rat liver homogenate, however, was found to hydrolyze cocarboxylase even aerobically, contrary to the results with pigeon liver.

After experimentation, it was determined that this difference was due to the state of division of the tissues, rat liver being more fragile than pigeon liver. If pigeon liver is sufficiently damaged, it can be made to behave as does rat tissue.

The experiments which led to this conclusion are described in this communication.

EXPERIMENTAL. Young albino rats bred at the laboratory were used. For experiments with pigeons those of the common variety which were apparently in good condition were obtained.

Homogenized tissue was prepared in a modified Potter and Elvehjem apparatus obtained from the Scientific Glass Apparatus Company

The final concentration of thiamin was 100 γ per cubic centimeter, and that of sodium pyrophosphate, 0 01 M   In most experiments Ringer phosphate buffer was used, but in a few experiments (Table IV) bicarbonate and barbital buffers were used   These did not modify the results appreciably

Experiments were usually carried out in Warburg manometers   the vessels of which had a capacity of about 30 cc   Occasionally anaerobic experiments were done in Thunberg tubes   The temperature was 37°C in all cases   At the end of the experimental period the vessels containing the tissue were placed in ice

TABLE I

Pigeon liver

Tissue was suspended in 2 cc Ringer phosphate buffer pH 7 5

TISSUE	GAS	COCARBOXYLASE IN γ PER GRAM FRESH TISSUE				
			After 60 minutes			
		Start	No addition	Thiamin	Pyrophosphate	Thiamin + pyrophosphate
500 mg slices	O_2	6 4	6 0	7 6		
	N_2	6 4	3 0	3 8		
500 mg homogenate (1 min)	O_2	9 8	9 0	8 1	9 1	11 2
	O_2	5 3	5 0	5 6	4 6	8 0
	N_2	5 3	2 0	3 4	4 6	5 2
	O_2	10 9	10 8	10 4	9 4	13 8
250 mg slices	O_2	9 1	9 2	14 6	9 6	18 6
	N_2	9 1	6 8	8 2	10 1	10 6
225 mg slices	O_2	11 8	9 3	13 8	11 4	13 4
200 mg slices	O_2	9 5	11 0	18 2	12 5	11 5
300 mg slices	N_2	10 3	8 5	8 2	6 3	6 4

In the experiments in which homogenate was used the tissue suspension was washed as quickly as possible into centrifuge tubes diluted to 10 cc and heated at 100°C for 5 minutes to destroy the enzymes   In the case of experiments with slices the tissue was homogenized, then treated as described above   The denatured tissue suspensions were centrifuged and the supernatant fluid used for cocarboxylase determinations   Cocarboxylase was determined by a manometric method, using the split enzyme prepared from yeast by the method of Green et al (3)

RESULTS AND DISCUSSION   On repeating Ochoa s experiments we obtained results identical with his, both aerobically and anaerobically if slices of pigeon liver were used or if the tissue was homogenized for a short time only (Table I)

If, however, the liver was submitted to prolonged homogenization (5 minutes), it was found that such tissue hydrolyzed cocarboxylase in an atmosphere of

TABLE II

500 mg. homogenized pigeon liver in 2 cc. Ringer-phosphate buffer in atmosphere of oxygen

TIME OF HOMOGENIZATION	COCARBOXYLASE IN γ PER GRAM FRESH TISSUE				
	Start	After 60 min. incubation			
		No addition	Thiamin	Pyrophosphate	Thiamin + pyrophosphate
minutes					
1.5	9.8	9.0	8.1	9.1	11.2
5.0	9.8	3.8	3.0	8.2	8.4
1	10.9	10.8	10.4	9.4	13.8
5	10.9	8.9	8.1	9.0	8.3

TABLE III

Rat liver slices

Slices suspended in Ringer-phosphate buffer. Total volume 2.2–2.4 cc.

WT. OF TISSUE	GAS	COCARBOXYLASE IN γ PER GRAM FRESH TISSUE				
		Start	After 60 minutes			
			No addition	Thiamin	Pyrophosphate	Thiamin + pyrophosphate
mg.						
500	O₂	7.6	7.6	8.9		
	N₂	7.6	7.3	7.2		
500	O₂	11.0	6.6	13.3	12.6	20.0
	N₂	11.0	8.4	7.4	10.4	13.4
500	O₂	13.9	9.7	21.7	10.8	24.2
	air	13.9	8.6	13.4	8.0	12.8
300	N₂	12.5	11.1	10.7	14.6	12.2
	O₂	17.0	14.2	18.9	15.6	23.2
	air	17.0	15.8	21.2	20.0	23.3
300	N₂	12.4	10.4	8.8	11.9	10.4
400	O₂	17.0	13.4	18.9	13.5	21.0
275	O₂	14.2	11.6	21.3	8.2	12.4

oxygen which was the reverse of the results found in sliced liver or in liver submitted to short homogenization (Table II).

On carrying the studies further in the rat the same reaction was found in sliced liver but even one minute homogenization of the tissue destroyed its ability to

synthesize cocarboxylase from thiamin in oxygen (Tables III and IV)   It was further observed that in rat liver, which is very fragile, it was difficult to make slices without obtaining some degree of hydrolysis in control vessels which did not contain thiamin or pyrophosphate

Thus one may say that in general in relatively intact tissue in oxygen, synthesis of cocarboxylase occurs, whereas in the same preparation in nitrogen hydrolysis takes place   On the other hand, severely damaged tissue shows hydrolysis of cocarboxylase both aerobically and anaerobically   One may then

TABLE IV

Homogenized rat liver

500 mg homogenized rat liver in a total volume of 2 4 cc

GAS	BUFFER	Start	COCARBOXYLASE IN γ PER GRAM FRESH TISSUE			
			After 60 minutes			
			No addition	Thiamin	Pyrophosphate	Pyrophosphate + thiamin
air	Bicarbonate	22 4	5 4	5 2		
N₂	Bicarbonte	23 4	13 2	14 8		
O₂	Ringer-phosphate	15 1	6 4	6 5	9 8	10 4
N₂	Ringer-phosphate	18 0	12 8	10 4		15 6
air	Barbital	21 8	12 6	12 8		
N₂	Barbital	21 8	15 2	18 4		
N₂	Ringer-phosphate	18 9	14 6	14 4	16 6	16 7
O₂	Ringer-phosphate	15 3	8 1	10 5		
N₂	Ringer-phosphate	27 6	14 2	16 8	17 2	18 0
O₂	Ringer-phosphate	18 5	9 4	11 0	13 0	16 2
N₂	Ringer-phosphate	15 5	11 5	12 0	14 5	14 1
O₂	Ringer-phosphate	13 6	10 9	12 8	13 8	14 4

conclude that the ability to synthesize cocarboxylase would seem to depend on the integrity of the cell (or of some essential enzyme systems) as well as on the conditions for active oxidative metabolism

This difference between the behavior in oxygen of slices and that of homogenate may be seen graphically in figures 1 and 2   In figure 1 the synthesis of cocarboxylase by sliced rat liver reaches a maximum in about two hours after which hydrolysis is the dominant factor   As may be seen in figure 2, with homogenized rat liver the rate of synthesis exceeds that of hydrolysis during only the first fifteen minutes

As phosphate is an essential component of this reaction pyrophosphate was added alone and together with thiamin. It will be seen that pyrophosphate has little effect on the synthesis of cocarboxylase by itself, but when a large amount of thiamin is present it may greatly increase the degree of synthesis. It would seem that the giving of pyrophosphate might be indicated when large amounts of thiamin are being administered.
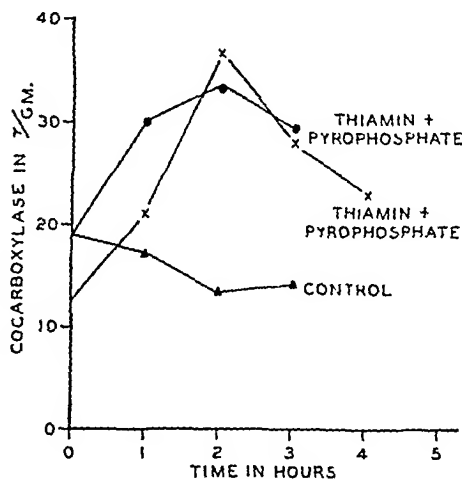
FIG. 1. SYNTHESIS OF COCARBOXYLASE BY RAT LIVER SLICES
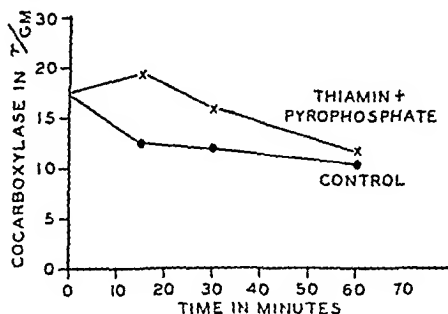
FIG. 2. SYNTHESIS OF COCARBOXYLASE BY HOMOGENIZED RAT LIVER

SUMMARY

1. The synthesis and hydrolysis of cocarboxylase by pigeon and rat liver in several states of dispersion have been investigated.

2. Ochoa's observations, that pigeon liver slices and homogenate can synthesize cocarboxylase from thiamin aerobically and that the same tissue dephosphorylates cocarboxylase anaerobically, have been confirmed.

3. The addition of pyrophosphate with thiamin to liver tissue may result in synthesis of cocarboxylase greater than that which occurs on the addition of thiamin alone.

4. The amount of hydrolysis of cocarboxylase by liver tissue varies directly with the severity of damage to the tissue.

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STUDIES ON SHOCK INDUCED BY HEMORRHAGE

VI. Cocarboxylase Phosphatase in Dog Serum[1]

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It has been observed (1) that serum contains no cocarboxylase. In our previous studies on shock induced by hemorrhage we have administered cocarboxylase to dogs in shock, occasionally with very marked beneficial results, at other times with beneficial results similar to those seen after thiamin administration. While carrying out the analyses reported in the previous paper we confirmed the absence of cocarboxylase in serum. The usual explanation of this phenomenon is that the cocarboxylase is dephosphorylated by an intracellular phosphatase and that thiamin alone, being more diffusible than cocarboxylase (2), then diffuses into the plasma.

However, on investigating the hydrolysis of cocarboxylase in shock previously reported, we found that in most samples of dog serum tested a very active phosphatase is present which hydrolyses cocarboxylase so that if any cocarboxylase did diffuse into the plasma it would be rapidly dephosphorylated. On this basis treatment of shock with cocarboxylase would not be of greater benefit than therapy with thiamin. Some of the characteristics of this phosphatase are presented here.

EXPERIMENTAL. Fresh dog blood was allowed to clot and the serum was separated by centrifugation. Barbital buffer at pH 7.4 was used unless otherwise stated.

Buffer. Phosphate M/15, borate M/15, barbital M/20, all at pH 7.4 were tested. Three cc. serum, 3 cc. buffer or H_2O and 1 cc. of cocarboxylase solution (40 γ) were incubated in test tubes at 37°C. One cc. samples were removed at 15, 30 and 60 minute intervals, diluted to 10 cc. and heated at 100°C for 5 minutes. Cocarboxylase determinations were done as described previously. The results are shown in figure 1. Barbital had no effect on the phosphatase activity, borate inhibited somewhat and phosphate inhibited considerably.

pH. Tubes containing 1 cc. serum, 5 cc. barbital buffer at different hydrogen ion concentrations, and one mg. cocarboxylase were incubated at 37°C. One cc. samples were removed at 5, 15 and 30 minutes after the start of the experiment. The results are shown in figure 2. The activity of the phosphatase increased with increasing pH between 7 and 8.5. Above pH 8.5 the spontaneous

hydrolysis of cocarboxylase was such that an accurate determination of hydrolysis due to the enzyme could not be obtained

Variation in activity of the phosphatase with concentration of cocarboxylase.
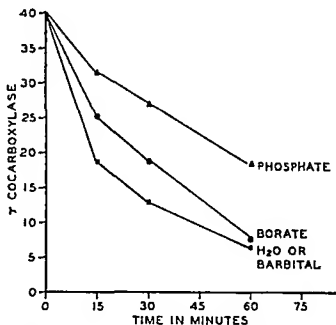
FIG 1. EFFECT OF BUFFERS ON THE RATE OF HYDROLYSIS OF COCARBOXYLASE BY SERUM PHOSPHATASE
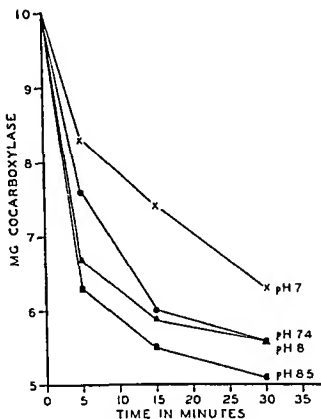
FIG 2 EFFECT OF pH ON THE RATE OF HYDROLYSIS OF COCARBOXYLASE BY SERUM PHOSPHATASE

Tubes containing 3 cc. serum, 3 cc. barbital buffer and 1 cc. cocarboxylase solution were incubated at 37°C. and one cc. samples were removed at 15, 30 and 60 minutes after the start of the experiment. The activity of the phosphatase was highest with the highest concentration of cocarboxylase. It is possible that

TABLE I

Influence of concentration of cocarboxylase on rate of hydrolysis

COCARBOXYLASE			
At start	After 15 minutes	After 30 minutes	After 60 minutes
11.9 mg.	9.5 mg.	9.5 mg. (?)	8.4 mg.
946 γ	701 γ	526 γ	245 γ
44 γ	40 γ	31 γ	16 γ
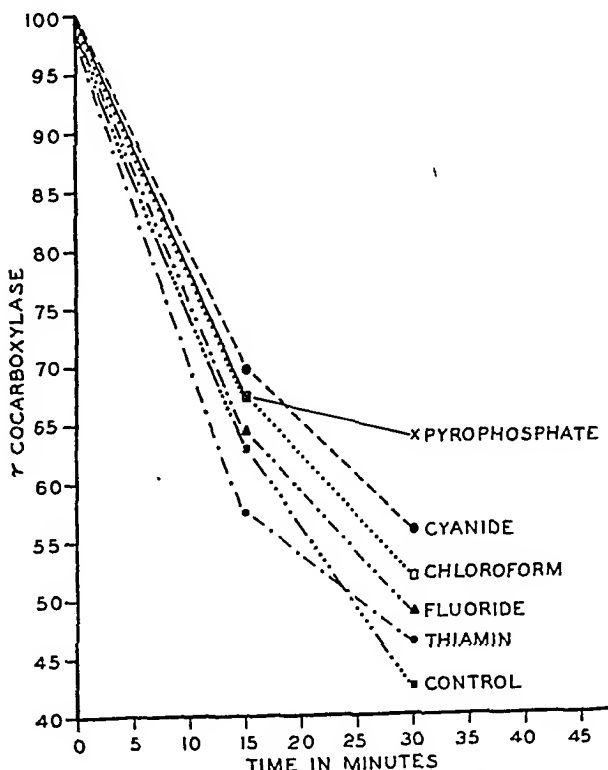
FIG. 3. EFFECT OF ADDED SUBSTANCES ON SERUM PHOSPHATASE

complete saturation of the enzyme was not attained even at this concentration, but the results give some idea of the rate at which cocarboxylase can be hydrolyzed by serum. The rate of hydrolysis fell off more rapidly with time in the presence of the larger amounts of cocarboxylase.

Effect of added substances. Tubes containing one cc. serum, 3 cc. barbital buffer and 100 γ cocarboxylase were incubated and one cc. samples were removed at 15 and 30 minutes after the start of the experiment. The results of such an experiment are given in figure 3. Westenbrink et al. (3) found in studies of yeast phosphatase that the enzyme was inhibited by thiamin in fairly low concentrations. However, the serum phosphatase was not appreciably affected by thiamin in concentrations as high as 17 mg. per cc. Pyrophosphate in .01 M concentrations inhibited the phosphatase, and cyanide in .08 M inhibited to about the same extent. NaF (M/40) and $CHCl_3$ (1 cc. saturated H_2O solution) inhibited slightly. Figure 3 shows the effect of some inhibitors. Phloridzin in a final concentration of .01 M had no effect.

SUMMARY

1. Dog serum contains a phosphatase which hydrolyses cocarboxylase.
2. Its activity increases with increase of pH from 7–8.5.
3. It is inhibited by cyanide, pyrophosphate, and slightly by NaF and $CHCl_3$.

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THE EFFECT ON THE FETUS OF PENTOBARBITAL SODIUM AND PENTOTHAL SODIUM

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Pentobarbital sodium and pentothal sodium find such extensive use in obstetrical practice that it is desirable to determine their effects on the fetus. The experiments reported here were undertaken to study the effects of these drugs on the respiration of the rabbit fetus, and to ascertain whether the effects are direct or are due to changes in oxygen saturation in the blood.

METHOD AND MATERIAL. Rabbits were prepared for observation of fetal respiration by the technique of Rosenfeld and Snyder (6). The respiratory movements of the fetuses were observed directly through the wall of the uterus following laparotomy carried out beneath the surface of a saline bath. The barbiturates were injected into the maternal ear vein at the rate of 1 cc. per minute, using a 5% solution in distilled water.

In 8 litters, in which the depressant effect of pentobarbital sodium was studied, 21 fetuses were observed and 43 living fetuses were delivered at the end of the experiments. In 8 other litters, in which the effect of pentothal sodium was investigated, 20 fetuses were observed and 51 living fetuses were delivered.

OBSERVATIONS. 1. *Pentobarbital sodium.* Direct observation of full-term fetuses within the intact uterus revealed a striking effect upon fetal respiratory movements following the administration of pentobarbital sodium to the maternal animal. Depression or disappearance of respiratory movements was regularly noted. Furthermore, the magnitude of the decrease in rate of respiration and the duration of effect were related to the quantity of the drug which had been administered. At a dosage level of 20 mg./kg. maternal body weight, the depression of fetal respiration was marked, falling to one-third or less of the initial rate, and usually persisted longer than one-half hour. With 10 mg./kg. a decrease in fetal respiratory rate occurred but did not approach the level of apnea. Usually within fifteen minutes the rate returned to normal. After 5 mg./kg. the decrease was smaller and less prolonged than that following 10 mg./kg. In addition to the immediate response of the fetuses, it was evident that preceding doses, even of 5 mg./kg., influenced the amount and duration of the depression of fetal respiratory activity which followed subsequent injections. Thus, in rabbit 307, fetal apnea followed a total dose of 20 mg./kg. (fig. 1). An initial dose of 5 mg./kg. resulted in a definite decrease in respiratory rate and, likewise, a second injection of 5 mg./kg. slowed respiration. The duration of the respiratory depression was about ten minutes. An addition 10 mg./kg. dose, injected an hour later, resulted in fetal apnea and a slower rate of fetal respiration in the subsequent hour than in the

[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Chicago.

preceding one, thus indicating a cumulative effect of the previous injections
The maternal animal showed a little analgesia following 10 mg /kg , although the
respiratory rate was diminished   After 20 mg /kg , the respiratory rate was
considerably slower and the response to nasal stimulation was sluggish, although
the conjunctival reflex remained active

In table 1 are recorded the results of experiments in 8 litters of rabbits in which
fetal respiratory activity continued throughout a period of 100 to 200 minutes
Doses of 5 or 10 mg /kg of pentobarbital sodium were injected into the maternal
ear vein at various intervals, usually about 20 minutes apart   Under these
conditions, it was found that at a dosage level of 20 mg /kg fetal respiration was
decreased below a level of one third of the rate which preceded injection, and the
duration of depression was usually thirty minutes or longer
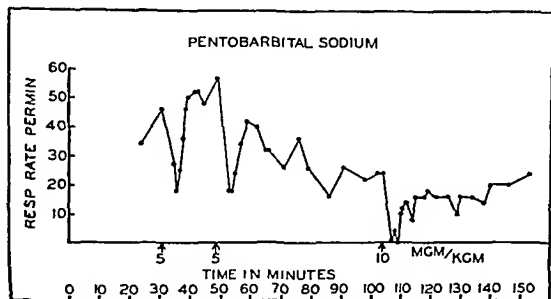
FIG 1 The effect upon fetal respiratory activity of pentobarbital sodium showing that
depression deepens as total dosage rises   Intravenous injection into maternal animal at
full term at rate of 17 mg /kg per minute   Rabbit No 307

The total dosage of pentobarbital sodium during an entire experiment did not
exceed 30 mg /kg   At this dosage level, rhythmical fetal respiration was
markedly depressed or abolished, and this effect persisted until the end of the ex
periment   The maternal animal showed slightly greater depression than after a
20 mg /kg dose

2 *Pentothal sodium*   Observation of the effect of pentothal sodium on the
fetus afforded additional evidence of the striking depression of fetal respiratory
activity following the administration of a barbiturate, although pentothal sodium
is inactivated by the body more rapidly than pentobarbital sodium   In a
typical experiment (fig 2, rabbit 41), it was evident that following pentothal
sodium the rate of fetal respiration was markedly decreased, but the duration of
depression was briefer than in the case of pentobarbital sodium   About five
minutes following the injection of the second and third doses of 10 mg /kg each,
respiratory movements had reached the pre injection level   Despite the ad

ministration of a total of 45 mg./kg. of pentothal sodium within an hour, rhythmical respiratory movements persisted at the end of the experimental period of

TABLE 1

Effect of pentobarbital sodium on intrauterine respiration

A single fetus of a litter was selected for tabulation of the record of changes of respiratory rate.

RABBIT NUMBER	TIME OF INJECTION OF MATERNAL ANIMAL (MIN. AFTER LAPAROTOMY)	DOSAGE OF PENTOBARBITAL SODIUM INTRAVENOUSLY	FETAL RESPIRATION BEFORE INJECTION	FETAL RESPIRATION AFTER INJECTION	DURATION OF RESPIRATORY DEPRESSION	DEPRESSANT DOSE (RESP. DECREASED TO ½ INITIAL RATE)	DURATION OF EXPERIMENT
		mg./kg.			min.	mg./kg.	min.
307	34	5	40	20		20	160
	52	5	50	20			
	106	10	25	5	30		
463	21	10	45	5	40	10	180
	122	10	25	5	50		
300	22	5	50	30		20	120
	33	5	45	30			
	48	10	25	5	10		
	72	5	30	5	50		
382	15	10	40	25		20	200
	37	10	60	20	40		
	88	10	10	0	40		
604	19	10	35	25		30	130
	35	5	25	10			
	59	5	20	15			
	82	5	25	10			
	90	5	25	5	30		
710	19	5	20	15		20	140
	29	5	10	10			
	39	5	15	10			
	49	5	15	5	30		
	84	5	5	0	25		
812	13	10	30	15		20	120
	34	10	20	5	30		
	64	10	35	15	40		
733	19	10	30	15		20	110
	37	10	20	5	10		
	62	10	20	5	40		

one and one-half hours.   The maternal animal showed little or no analgesia at the end of the experiment.   However, immediately following injection at the level of

20, 30 and 45 mg./kg., this animal showed marked slowing of respiration, and definite analgesia was revealed by sluggish response to nasal and conjunctival stimulation. After five minutes, depression of the maternal animal had largely disappeared.

Table 2 records the results of experiments with 8 litters in which intra-uterine respiratory activity continued for periods of 120 to 270 minutes. During this time the influence on the fetus of the various amounts of pentothal sodium was observed. Pentothal sodium, in single doses of 2.5 to 15 mg./kg. maternal body weight, was injected into the maternal ear vein at intervals of about fifteen minutes. Under these conditions it was found that a single injection of 10 mg./kg. of pentothal sodium decreased fetal respiratory activity, the rate being lowered usually below a level of one-third that of the initial rate. Recovery required five to ten minutes. No marked analgesia of the maternal animal was
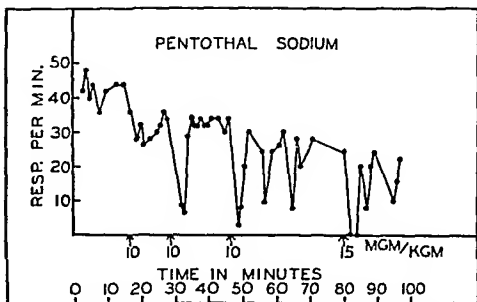
Fig. 2. ... ... nal sodium showing that Intravenous injection of Rabbit No 41.

noted following a single dose of 10 mg./kg. of pentothal sodium, although the respiratory rate was diminished.

The total dosages in various animals ranged from 30 to 55 mg./kg. during an experiment. There was evidence of relatively little cumulative effect of pentothal sodium upon the fetuses in contrast to that of pentobarbital sodium. At the end of an experiment, the fetal respiratory rate remained within normal limits. The outstanding feature was the brief duration of the depression of fetal respiration.

3. *The oxygenation and carbon dioxide content of fetal blood during apnea following the administration of pentothal sodium.* The occurrence of apnea in the fetus, despite the persistence of normal respiration in the maternal animal following administration of barbiturates, afforded a favorable opportunity to determine whether or not the oxygen and carbon dioxide content of the fetal blood remained

TABLE 2

Effect of pentothal sodium on intrauterine respiration

RABBIT NUMBER	DEPRESSANT DOSE (RESP. DECREASED TO ½ INITIAL RATE)	FETAL RESPIRATION AT BEGINNING	FETAL RESPIRATION AFTER FIRST DEPRESSANT DOSE	DURATION OF RESPIRATORY DEPRESSION	TOTAL DOSE OF PENTOTHAL SODIUM GIVEN MATERNAL ANIMAL INTRAVENOUSLY	NUMBER OF DOSES	DURATION OF EXPERIMENT
	mg./kg.			*min.*	*mg./kg.*		*min.*
413	15	25	0	5	40	7	170
459	25	70	20	5	45	6	130
41	20	45	10	5	45	4	120
439	10	35	5	10	55	5	120
467	5	20	5	5	30	6	170
753	10	35	5	5	40	4	170
453	10	25	5	10	30	6	150
431	5	40	5	5	30	12	280

TABLE 3

Fetal blood oxygen and carbon dioxide during anesthetic apnea

RABBIT	FETUS NUMBER	TIME	RESPIRATORY RATE	OXYGEN CAPACITY	OXYGEN CONTENT	OXYGEN SATURATION	CARBON DIOXIDE CONTENT	DOSE OF PENTOTHAL TO MATERNAL ANIMAL I.V.
		minutes		*vol. %*	*vol. %*	*%*		*mg./kg.*
516	F_1	0	86	13	11	85	51	
		23*						15
	F_1	27	0	12	11	* 92	42	
	F_1	50	72	11	10	91	29	
	F_2	86	20	14	13	93	41	
		118*						10
	F_2	121	0	13	10	77	30	
522	F_1	0	40	13	11	85	40	
		20*						15
	F_1	25	0	13	9	69	40	
412	F_1	0	66	15	10	67	30	
		19*						15
	F_1	21	0	13	10	77	25	
590	F_1	0	30	12	10	83	38	
		28*						20
	F_1	33	0	11	8	72	37	
	F_1	50	54	11	9	82	35	
	F_2	90	20	13	11	85	36	
		123*						10
	F_2	125	0	13	12	92	28	

* Injected.

normal during the interval of apnea.   In order to obtain blood samples from the umbilical vein, the uterus was opened through a small incision and fetuses were extruded into the saline bath, the umbilical circulation remaining intact.   The fetuses remained submerged beneath the surface of the bath so that no air entered the respiratory tract.   The oxygen content, carbon dioxide content (11) and hemoglobin concentration (12) were determined in blood samples of 0.25 cc. from the umbilical vein.   The oxygen capacity of the blood was calculated from the hemoglobin values.

Observations were made on seven fetuses obtained from four litters at term in which rhythmical respiratory movements were abruptly interrupted and apnea
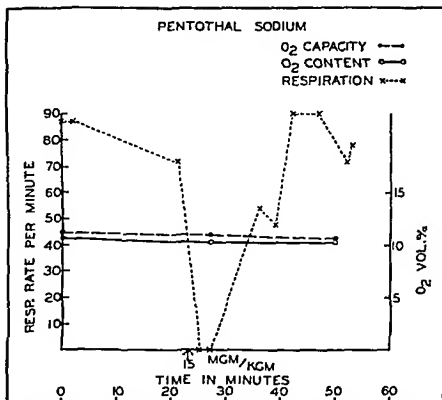
Fig. 3. Anesthetic apnea. In a full term fetus the rhythmical ...

occurred following injection of 10 to 20 mg./kg. of pentothal sodium into the maternal ear vein (table 3). Umbilical vein blood samples were taken before, during, and after the fetal anesthetic apnea. No evidence of anoxemia was observed (fig. 3).

The oxygen content of the blood of fetuses during the apnea which followed the administration of pentothal sodium was 8 to 12 volumes per 100 cc. of blood, and the oxygen saturation was 69 to 92 per cent. In blood obtained about one-half hour preceding the injection of pentothal sodium, while the fetuses were showing rhythmical respiratory movements at a rate of 20 to 86 per minute, the oxygen content was 10 to 13 volumes per 100 cc. of blood, and the oxygen saturation of the blood was 67 to 93 per cent. In two fetuses, a third blood sample was ob-

tained following recovery from apnea at 17 and 23 minutes after injection when the respiratory rate was 54 and 72 per minute respectively. In the latter samples the oxygen content was 9 and 10 volumes per 100 cc. of blood, and the oxygen saturation was 82 and 91 per cent.

There was no evidence that the carbon dioxide content of the blood in these experiments was related to apnea of the fetus.

DISCUSSION. The widespread use of barbiturates during the first stage of labor has prompted numerous attempts to evaluate the effects of these drugs upon mother and child (1, 2, 3, 4). Many factors, such as trauma, anoxemia, and anesthetics, complicate passage through the birth canal and may result in injury of the fetus. It has been difficult to isolate the factor of anesthesia under clinical conditions and to determine the role of barbiturates in the pathogenesis of respiratory failure at birth.

In the present experiments full-term fetuses were under direct observation before the onset of labor, in contrast to clinical studies based upon the state of the fetus following delivery. The changes in rate of intrauterine respiratory movements afforded a means of measuring the effect of pentobarbital sodium and pentothal sodium upon the fetus. Likewise, the responses of the maternal animal were determined readily at frequent intervals following various doses of the drugs (10). No general anesthetic was given, since exposure of the uterus was carried out by laparotomy following section of the lumbar spinal cord under procaine anesthesia.

Under such favorable experimental conditions, it was found that 20 mg./kg. of pentobarbital sodium strikingly decreased fetal respiratory activity, while 30 mg./kg. resulted in prolonged respiratory depression. In the case of pentothal sodium, whose effect is known to be much briefer, 10 mg./kg. markedly reduced the rate of fetal respiration; however, after a dosage of 3 to 5 times this amount, fetal respiratory movements returned to normal within the experimental period.

In attempting to correlate the response to pentobarbital sodium in man and in the rabbit, it is of interest to note that the basal metabolic rate of the rabbit is 3 times greater than that of man, the oxygen consumption per kilogram per hour at best being about 200 cc. in man in contrast to 650 cc. in the rabbit (5). Thus, a total dose of 7 grains of pentobarbital sodium in a man of 70 kilograms amounts to 6.5 mg./kg. or, if multiplied by 3, is equal to the dose which resulted in marked depression of the fetal respiratory system in the rabbit when administered intravenously. In the case of pentothal sodium in a man of 70 kilograms, a total dose of 4 grains amounts to 3.7 mg./kg. or, if multiplied by 3, is equal to the dose which caused marked depression of fetal respiration in the rabbit when given intravenously at the rate of 17 mg./kg. per minute.

Pentothal sodium, because of its prompt action as an anesthetic and its rapid inactivation in the body, was favorable for investigating the nature of anesthetic apnea. The three types of apnea, namely, anoxemic, acapnic and anesthetic, may be induced experimentally in fetuses showing rhythmical respiratory movements (8). In the present experiments it was demonstrated that the apnea which followed injection of pentothal sodium was caused by the anesthetic agent rather

than by anoxemia or acapnia. Blood samples obtained from the umbilical vein during the interval of apnea, as well as before and after it, were normal. Furthermore, the abrupt onset of fetal apnea and the recurrence of regular respiratory movements were clearly parallel to the administration of the drug and the rate of its inactivation in the body. Thus, in the absence of anoxemia, fetal apnea occurred promptly following intravenous anesthesia; in contrast, in the absence of any anesthetic agent, as previous experiments have shown (9), sudden oxygen want resulted in prompt interruption of rhythmical fetal respiratory movements. Both types of apnea, anesthetic and anoxemic, may be transient and easily reversible under experimental conditions.

Finally, it may be pointed out that a complete assay of the action of pentobarbital and pentothal in obstetrical analgesia must include not only observation of the direct effect of the drugs upon the fetus but also determination of the effect upon the maternal organism during labor. The methods of analysis and results in the case of morphine have been described previously (7).

CONCLUSIONS

The effect upon the full-term rabbit fetus of various amounts of pentobarbital sodium and pentothal sodium and determined by the changes in rate of fetal respiratory movements following intravenous injection into the maternal animal.

1. Pentobarbital sodium in doses of 5 or 10 mg./kg. resulted in decrease of respiratory rate for less than 15 minutes; 20 mg./kg. resulted in depression of the fetal respiratory rate to about one-third of the initial level for about one-half hour; 30 mg./kg. deeply depressed or abolished fetal respiration for the duration of the experiment.

Following a dose of 20 mg./kg., there was no marked analgesia in the maternal animal.

2. Pentothal sodium, in doses of 10 mg./kg., decreased the fetal respiratory rate to one-third the initial level for about 5 minutes; after a total dosage of 30 to 55 mg./kg., relatively little cumulative effect upon the fetuses was noted.

Following 10 mg./kg., there was no deep analgesia in the maternal animal.

3. Fetal apnea following injection of pentothal sodium was caused by the drug and was not due to anoxemia since analyses of fetal blood showed that the oxygenation and carbon dioxide content remained normal.

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THE EFFECT OF HAEMORRHAGE ON ANAESTHETIC DOSAGE

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There is a clinical impression that a patient who has lost a considerable amount of blood is more than normally susceptible to anaesthesia (MacIntosh and Pratt (1), Harbord (2))

Experimental evidence to support this impression is scarce. Price et al (3) noticed that dogs anaesthetised with pentobarbitone showed a deepening of narcosis during and after haemorrhage. MacIntosh (4) found that haemorrhage of 20 c.c./kg bodyweight did not significantly reduce the dose of pentothal required to abolish the corneal reflex in cats, although the dose to produce respiratory failure was reduced by 30%. The safety ratio remained high in all animals. Bayliss (5) observed that a cat required less ether for maintenance of anaesthesia after experimental haemorrhage and McKeen Cattell (6) also showed that loss of 15-20% of the total blood volume rendered a cat more sensitive to ether

The work to be described in this paper records observations on the dose of anaesthetic agents required by comparable groups of normal animals and of animals previously subjected to moderate haemorrhage. The first part deals with pentobarbitone anaesthesia in rabbits, the second part with ether anaesthesia in rats

1 *Pentobarbitone in rabbits*

METHOD Rabbits weighing 1 5 to 3 0 kg (average 2 1 kg) were used unselected for sex apart from the rejection of pregnant females Pentobarbitone was injected as a 2% solution in distilled water into the ear vein The animal was bled by puncture of the ear vein, the ear being warmed to ensure vasodilatation 15 or 20 c.c of blood per kg body weight was withdrawn in this manner in an average time of 32 minutes Pentobarbitone was injected into bled animals 2½ hours after haemorrhage, since it had been found in earlier experiments that haemodilution was practically complete by this time

The anaesthetic dose The solution was slowly injected at the rate of 0 1 c.c (= 2 mg. pentobarbitone), every minute This slow rate ensures a slow and uniform rise in the concentration of pentobarbitone in the blood Thus the central nervous system never receives a massive dose causing sudden anaesthesia from which there is rapid recovery as the barbiturate is distributed throughout the body The injection was continued until the rabbit was no longer able to remain upright on the table but fell on one side with limb muscles relaxed The amount of pentobarbitone required to produce this arbitrary state of anaesthesia was taken as the "anaesthetic dose"

Recovery time When the anaesthetic dose had been determined, sufficient solution was injected during the next thirty seconds to bring the total dose to 1 c.c (= 20 mg pentobarbitone) per kg bodyweight, and the time of completion of the injection was noted The interval until the rabbit could regain the sitting position unaided was taken as the "recovery time"

Respiratory failure In a few animals after anaesthesia had been established, the injection was continued at the rate of 0 1 c.c of solution every 12 seconds When respirations occurred at intervals of more than 12 seconds, the time was noted together with the total of

259

solution injected. The injections were then continued until *no respiration had occurred for one minute*, the average of the total doses injected at the beginning and end of this minute being regarded as the dose producing respiratory failure.

Safety ratio. This is the ratio of the dose producing respiratory failure and the dose producing anaesthesia.

TABLE 1

Anaesthetic dosage of Pentobarbitone in rabbits

2% solution injected intravenously at 0.1 c.c. per minute

CONTROLS		AFTER HAEMORRHAGE OF 15 C.C./KG.	
Weight	Anaesthetic dose	Weight	Anaesthetic dose
kg.	*mg./kg.*	*kg.*	*mg./kg.*
1.75	13.7	1.95	8.8
1.60	13.8	1.70	8.2
1.93	13.4	1.98	19.2
1.52	13.8	2.30	14.8
1.68	12.0	2.47	12.2
1.78	14.6	1.77	14.6
1.67	10.8	1.80	14.4
2.72	14.0	2.09	12.4
1.75	15.0	AFTER HAEMORRHAGE OF 20 C.C./KG.	
1.75	13.8		
2.06	15.4	2.03	16.4
2.56	13.4	2.80	7.8
2.70	14.0	2.15	10.2
1.86	16.1	1.94	11.6
2.15	13.9	1.85	12.0
2.17	15.4	2.05	11.8
2.17	21.2	1.83	11.0
1.67	13.2	1.80	10.0
2.17	11.1	1.70	12.9
3.20	18.8	2.03	10.8

RESULTS.   *Anaesthetic dose.*   The following values for the mean anaesthetic dose of pentobarbitone were obtained:

In normal animals....................................... 14.4 ± 0.5 mg./kg. (20)

2½ hrs. after haemorrhage of 15 c.c./kg............... 13.3 ± 1.3 mg./kg. (8)

2½ hrs. after haemorrhage of 20 c.c./kg.............. 11.5 ± 0.7 mg./kg. (10)

The number of animals used is given in brackets and table 1 shows the individual observations.

The difference in the anaesthetic dosage after haemorrhage of 15 c.c./kg., is not statistically significant ($P = 0.35$), but that after haemorrhage of 20 c.c./kg. is significant ($P = 0.05$).   This is a reduction of 18% in the anaesthetic dose.

Recovery time.   The mean recovery times were as follows:

In normal animals..................... .... 42 ± 6.6 mins. (6)

In animals bled 15 c.c./kg................................... 52 ± 9.7 mins. (7)

In animals bled 20 c.c./kg................................... 43 ± 7.1 mins. (7)

None of these differences is significant.

Respiratory failure   Three normal rabbits required 68, 44 and 75 mg /kg pentobarbitone to cause respiratory failure while three animals bled 20 c c /kg required 43, 52 and 47 mg /kg   The mean doses were 63 and 48 respectively, the 24% reduction being statistically significant   (See table 2)

Safety ratio   In the three normal rabbits this was 5 2, 4 0 and 4 0 (mean 4 4), and in the three bled animals 4 3, 4 0 and 4 4 (mean 4 2)   Although there was a 24% reduction in the lethal dose of pentobarbitone after haemorrhage of 20 c c /kg the safety ratio was not altered

2   Ether in rats

METHOD   White rats of both sexes weighing from 110 g to 310 g (average 180 g) were employed   Blood was removed by cardiac puncture, 0 6 to 2 6 c c /100 g bodyweight (average 1 5 c c /100 g), being obtained   It was found that no more than two attempts at cardiac puncture could be made without the risk of causing intrathoracic haemorrhage   A

TABLE 2

WEIGHT	ANAESTHETIC DOSE	RESPIRATORY FAILURE DOSE	SAFETY RATIO
(a) Normal rabbits			
kg	*mg /kg*	*mg /kg*	
1 67	13 2	68 2	5 2
2 17	11 1	44 4	4 0
3 20	18 8	75 0	4 0
Average 2 34	14 4	62 5	4 4
(b) Rabbits bled 20 c c /kg			
1 80	10 0	43 3	4 3
1 70	12 9	51 8	4 0
2 03	10 8	47 4	4 4
Average 1 84	11 2	47 5	4 2

number of unsuccessfully punctured animals was used as controls   It was found that their reaction was very similar to that of normal rats   Thus any differences found in bled rats were due to the loss of blood and not to the trauma   For anaesthetising the animals a system was employed consisting of a pump an Oxford vaporiser No 1 (7) and a specially constructed box   Ether air mixtures of various proportions were pumped to the box through the vaporiser at the rate of 5½ l per min   This flow supplied more than enough oxygen for six rats and ensured complete mixing of the contents of the box in about one minute   Usually 3 normal and 3 bled rats were anaesthetised simultaneously   From a side tube connected to the main tube delivering the mixture to the box samples could be taken for check determinations of the ether concentration being delivered using a simple Haldane gas analysis apparatus with sulphuric acid as the absorbing agent   Corrections were applied for the water vapour present   Though the vaporiser scale was previously calibrated for various tap positions check determinations were frequently carried out in the course of each experiment

Induction time   Six rats being placed in the box and the detachable side being screwed tightly in position, the pump was started   After a period of activity the

animals moved more slowly and after 3 to 5 minutes fell on to one side.   Gentle shaking of the box might at first elicit further movements, but in a few seconds there was no such response.   The time taken to reach this condition was taken as "induction time" and was recorded separately for each animal.   The end-point could be defined within 10 seconds.

Maintenance of anaesthesia.   When all the animals were anaesthetised, the ether concentration was reduced and the behaviour of the rats in this concentration noted for one hour.   Any major movements (but neglecting small head or limb movements), were taken to imply that anaesthesia was no longer maintained.

Recovery.   At the end of one hour's anaesthesia, all the rats were removed from the box and replaced in their cages.   Recovery time was taken as the interval until an animal was able to move on all fours.

RESULTS.   *Induction time.*   The time taken for induction of anaesthesia with different ether concentrations is shown in table 3.   The reduced induction times

TABLE 3

Effect of haemorrhage on induction times with ether

0.6–1.5 c.c./100 g. bodyweight =   9–23% of total blood volume
1.6–2 5 c.c./100 g. bodyweight = 25–38% of total blood volume

(assuming average blood volume 6 5 c.c./100 g.)

CONCEN-TRATION OF ETHER V/V	NORMAL RATS		BLED 0 6–1 5% OF BODYWEIGHT		BLED 1 6–2 0% OF BODYWEIGHT		TOTAL BLED RATS	
	Number	Induction time	Number	Induction time	Number	Induction time	Number	Induction time
7%	36	6'50" ±11"	20	5'00" ±16"	15	4'00" ±11"	36	4'30" ±12"
8%	64	5'00" ±10"	39	3'50" ±10"	15	3'15" ±17"	54	3'40" ±9"
9%	24	4'25" ±8"	9	4'20" ±14"	13	3'20" ±12"	24	3'45" ±11"

in bled animals are all significantly different from normal, not only after severer haemorrhages of 1.6 to 2.0 c.c./100 g., but also after relatively slight haemorrhage.

Maintenance of anaesthesia.   Concentration of 2.5 to 7% ether were employed for maintenance of anaesthesia and the results obtained are shown in figure 1.   In ether concentrations of less than 3%, anaesthesia was not maintained in any normal rats whereas some bled rats remained anaesthetised for the whole of one hour.   Higher ether concentrations (4 to 5%) maintained anaesthesia in only 43% of normal rats, whilst 75% of bled rats remained anaesthetised.   7% ether maintained anaesthesia in all normal and bled rats; 5 out of 9 rats in each group died during the hour.   From the graph it is seen that an ether concentration of 5–6% will maintain anaesthesia in 50% of normal rats, while only 3–4% is necessary for a similar effect in rats after haemorrhage.

When the number of rats actually anaesthetised was counted at 15-minute intervals during the hour of maintenance, results shown in figure 2 were obtained.

It can be seen that with a lower ether concentration (e g 3 5%), practically all the bled rats remained anaesthetised for the whole hour whereas a large proportion of normals were not anaesthetised for most of the time

DISCUSSION The experimental results obtained confirm the clinical impression that a bled animal is more susceptible to anaesthesia than a normal They also extend the work of MacIntosh (4) to different drugs and species

The two sets of experiments are not exactly comparable since we have no evidence of the extent of haemodilution which occurred in the rats, although time was allowed for the rabbits to make up their lost circulatory volume It would
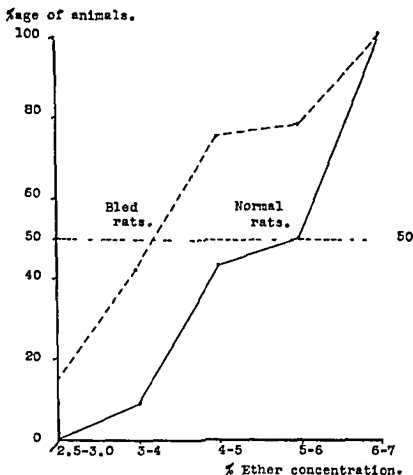
FIG 1 PERCENTAGE OF BLED AND NORMAL RATS REMAINING ANAESTHETISED FOR ONE HOUR IN DIFFERENT CONCENTRATIONS OF ETHER (v/v)

not be correct to assume from the results that since the reduction of pentobarbitone dosage in rabbits is relatively less than the reduction of the ether dosage in rats, a barbiturate is therefore a safer anaesthetic than ether for use in exsanguinated patients It is, however, pertinent to recall the work of Kendrick and Uihlein (8) who showed that the chance of shock was less under pentobarbitone than under ether Our experiments indicate that the safety ratio of pentobarbitone in rabbits is not altered by haemorrhage MacIntosh's results with pentothal in cats also suggest this Thus careful administration of a barbiturate is probably as safe in the depressed as in the normal patient Ether anaesthesia is, however, more readily controllable than the intravenous administration of

animals moved more slowly and after 3 to 5 minutes fell on to one side.   Gentle shaking of the box might at first elicit further movements, but in a few seconds there was no such response.   The time taken to reach this condition was taken as "induction time" and was recorded separately for each animal.   The end-point could be defined within 10 seconds.

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1.6–2.5 c.c./100 g. bodyweight = 25–38% of total blood volume

(assuming average blood volume 6.5 c.c./100 g.)

CONCEN-TRATION OF ETHER v/v	NORMAL RATS		BLED 0.6–1.5% OF BODYWEIGHT		BLED 1.6–2.0% OF BODYWEIGHT		TOTAL BLED RATS	
	Number	Induction time	Number	Induction time	Number	Induction time	Number	Induction time
7%	36	6'50" ±11"	20	5'00" ±16"	15	4'00" ±11"	36	4'30" ±12"
8%	64	5'00" ±10"	39	3'50" ±10"	15	3'15" ±17"	54	3'40" ±9"
9%	24	4'25" ±8"	9	4'20" ±14"	13	3'20" ±12"	24	3'45" ±11"

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c c /kg   This degree of haemorrhage reduced the dose producing respiratory failure by 24%, but the safety ratio was not altered.

3 Induction of ether anaesthesia in rats was more rapid after haemorrhage Loss of more than 25% of the blood volume reduced the induction time to 60, 65 and 75% of the normal time in ether concentrations of 7, 8 and 9%   Anaesthesia could be maintained in 50% of the bled rats by an ether concentration of 3 to 4%, while 5 to 6% ether was required to maintain anaesthesia in 50% of normal rats.

4 Recovery times after pentobarbitone anaesthesia in rabbits and after ether anaesthesia in rats were not significantly altered by haemorrhage

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STUDIES ON THE FATE OF HEROIN

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The chemical structure of heroin suggests a number of possible ways in which it may be excreted: in an unchanged form, as either monoacetyl derivative, as morphine, as a mixture, or as degredation products. Certain of these could be excreted in a conjugated form. Since heroin, like bound morphine, is readily hydrolyzed to morphine in a hot acid solution, it would be very difficult by present methods of chemical analysis to differentiate between the excretion of an acetylated morphine and its conjugated form.

The literature pertaining to the nature of the product excreted in urine after the administration of heroin hydrochloride contains conflicting reports. Sollmann (1) quotes Langer (2) that "the greater part of heroin is excreted unchanged in urine; some also in the feces. A part is destroyed and this destruction was said to be increased in habituation". Other investigators (3, 4) reported that the excretion product of heroin is morphine.

Goris and Fourmount (4) reported that an aqueous solution of heroin hydrochloride hydrolyzes at room temperature with a loss first of one and finally of both acetyl groups. Oberst and Andrews (5) found that the conductivity of a solution of heroin hydrochloride did not change from one minute to two weeks after preparation. Hence, hydrolysis must proceed at a slow rate in an *aqueous* lution at room temperature.

Wright (6) studied the rate of enzymatic hydrolysis of *monoacetylmorphine* and of heroin, using the Warburg technique. He was convinced that the purest available heroin hydrochloride contained some monoacetylmorphine hydrochloride. Chemical analysis of heroin for its percentage of carbon and hydrogen would not differentiate between monoacetyl-, diacety-morphine, *or a mixture of* these. Specific rotation of heroin hydrochloride also would not establish its composition.

The present study was undertaken to investigate further the chemical composition of heroin hydrochloride and to determine the amount and the chemical nature of the excreted product in addicted patients.

METHODS. It has been established that the addition of phenol reagents[1] and sodium carbonate to a solution of an opiate containing a free phenolic hydroxyl produces a blue color. Solutions of heroin hydrochloride[2] and 6-monoacetylmorphine hydrochloride[2] were

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[1] Phenol reagent, (Folin and Denis). This reagent is prepared as follows: 100 gm. sodium tungstate dissolved in 750 cc. water, 20 gm. phosphomolybdic acid, and 50 cc. 85 per cent phosphoric acid. The mixture is boiled for two hours and after cooling is diluted to one litre.

[2] Heroin hydrochloride (anhydrous) and 6-monoacetylmorphine hydrochloride · 2½H₂O were prepared by Dr. Lyndon F. Small, National Institute of Health, Bethesda, Md.

tested with sodium carbonate and phenol reagent (7) before and after acid hydrolysis (8)

Since an alkaline agent (sodium carbonate) and phenol reagent are used in the determination of morphine and monoacetylmorphine, the question is raised whether the blue color obtained with a solution of heroin hydrochloride by these reagents might not be due to hydrolysis of heroin by alkali   It has been found that phenol reagent reacts immediately with free phenolic hydroxyl groups (as in morphine) to form a blue color   The excess amount of this reagent rapidly becomes inactivated by the presence of alkali hence further addition of morphine or hydrolysis of heroin by sodium carbonate does not increase the intensity of the color   On the basis of this reaction it was possible to study the rate of hydrolysis of heroin in approximately 0.5 M sodium carbonate solution (26°C)

Solutions (25 cc) containing 1 0 mgm of heroin hydrochloride were placed in thirteen 50 cc volumetric flasks   To each flask 4 5 cc of saturated sodium carbonate were added by pouring as quickly as possible from a test tube   The phenol reagent was then added in like manner and the time between the two procedures recorded   The time allowed for hydrolysis for the various samples varied from 1 second to 10 minutes   Each flask was then filled to the 50 cc mark and allowed to stand 30 minutes before reading in a colorimeter against a standard morphine solution treated in a similar manner

Analyses were made for morphine (7, 8) in urine from three addicts receiving the minimal amount of morphine sulfate needed to satisfy physical dependence   Heroin hydrochloride was then substituted for morphine sulfate in amounts approximately one half as large and analyses were continued

For purposes of calculation it was assumed that the morphine like product in urine during both periods was morphine base   The total morphine base equivalent of anhydrous heroin hydrochloride after acid hydrolysis was 70 4 per cent

RESULTS   *Chemical analysis of 6 monoacetylmorphine and heroin*   *(a) 6 monoacetylmorphine*   This compound gives a color similar to, but slightly less intense, than that of morphine   Although 3 monoacetylmorphine was not available for testing, it may be assumed that it would not develop the color, since color development by the phenol reagent is through the free phenolic hydroxyl group (3 carbon)   Since the urinary residue from a patient receiving heroin hydrochloride gave a color before hydrolysis, the alkaloid could be either morphine, 6 monoacetylmorphine, or a mixture   Comparative color tests made on 6 monoacetylmorphine before and after hydrolysis showed only slight differences in the color intensity , insufficient to differentiate between the hydrolyzed and unhydrolyzed forms

(b) Heroin   It was found that an aqueous solution of heroin hydrochloride (1 mgm) developed a blue color when mixed with sodium carbonate and phenol reagent   This might indicate either that impurities were present in the heroin hydrochloride or that some hydrolysis takes place before complete development of the blue color

The rate of hydrolysis of heroin by sodium carbonate (26°C) is very rapid for the first two minutes (fig 1)   Approximately 58 per cent of the total sample was hydrolyzed (calculated as morphine base) in 2 minutes and 88 per cent in 10 minutes   Extrapolation of the curve to zero time gave a value of about 4 per cent as free morphine in the sample   This indicates that not less than 96 per cent of the heroin hydrochloride tested was diacetylmorphine hydrochloride   The conclusion seems warranted that most of the blue color obtained from a solution of heroin hydrochloride after the addition of the color reagent is due to the rapid hydrolysis of heroin by the sodium carbonate used in the test

Tests for the presence of acetyl groups in urinary extracts. Urine (130 ml.) from an addict in whom heroin hydrochloride had been substituted for morphine sulfate for 7 days was saturated with sodium carbonate and extracted in a separatory funnel with ethyl acetate at room temperature. The residue obtained after the evaporation of the solvent was further purified by acid and alkaline extractions and then divided into two equal portions. One portion was boiled for two hours in acid under a reflux condenser. These two fractions when analyzed for color intensity showed practically no difference, indicating that the extracted product contained a free phenolic hydroxyl group.
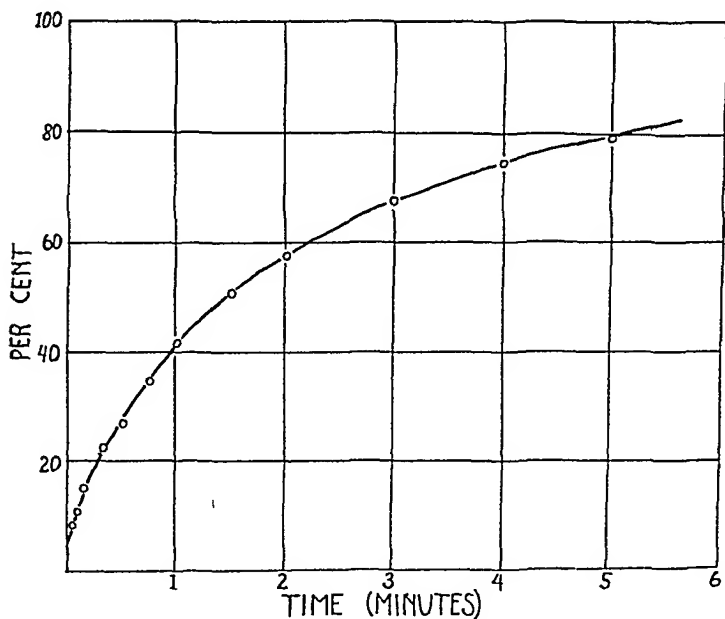
FIG. 1. RATE OF HYDROLYSIS OF HEROIN AT 26°C. IN .5 M Na₂CO₃ SOLUTION

Urine containing added amounts of heroin hydrochloride and extracted by the usual procedure in a liquid-liquid extractor, followed by acid and alkaline extractions, gave an intense blue color when the reagents were added, indicating that at least some of the diacetylmorphine had been hydrolyzed by this extraction technique.

Recently Wright (6) has shown that rabbit and human sera deacetylate heroin. Serum from all rabbits tested removed the 3-carbon acetyl group, but serum from some rabbits removed the 6-carbon acetyl group as well. A purified residue extracted from urine with ethyl acetate tested by Dr. Wright showed that no acetyl groups were present. A portion of the same residue was studied by Dr. S. T. Gross at the University of Illinois (9) for an X-ray diffraction pattern; he reported the presence of morphine in the sample.

These studies do not show whether diacetylmorphine was originally present in urine or whether it is excreted as one of its hydrolyzed products

Excretion studies in addicts receiving heroin hydrochloride   The daily amounts and the corresponding per cent of free and bound morphine excreted in the urine of morphine addicts before and after heroin hydrochloride substitution are presented in table 1   The per cent of free and bound morphine excreted in the urine of morphine addicts before substitution was approximately the same as in previous studies (8)   Half as much heroin hydrochloride as morphine sulfate satisfied physical dependence   The average amount of excretion of *free* morphine was proportionately decreased   The percentage excretion values were about the same, 7 0, 6 4 for morphine and heroin respectively   On the first day of substitution the percentage value was quite high, presumably due to "carry over" of morphine base from the morphine administration   The average daily amount

TABLE 1

The excretion of free and bound morphine (base) during morphine and heroin administration

PATIENT NO	DRUG USED	NO OF DAYS STUDIED	AV 24 HR URINE VOL	AVERAGE DAILY DOSE		AVER DAILY EXCRETION (BASE)				AVERAGE DAILY RATIO OF BOUND TO FREE MORPHINE
				Alka loidal salt	Equival morphine base	Free		Bound		
						mgm	%	mgm	%	
			cc	*mgm*	*mgm*					
362	M	7	2218	200	150 5	9 5	6 3	70 9	47 1	7 4
	H	14	1853	119	82 7	4 5	5 5	40 7	51 7	9 7
492	M	8	1265	200	150 5	11 1	7 4	69 6	48 8	7 3
	H	8	1075	101	72 5	5 5	7 6	39 7	55 0	8 4
493	M	6	1280	200	150 5	10 6	7 1	70 8	47 2	6 9
	H	9	1029	97 8	70 4	4 9	6 8	33 2	45 0	6 9
Average	M	21	1595	200	150 5	10 5	7 0	70 4	47 8	7 2
	H	31	1402	108	76 5	4 9	6 4	38 3	50 9	8 5

of *bound* morphine in urine of these patients during the morphine administration was slightly less than twice the amount during the heroin administration, the values being 70 4 and 38 3 mgm , respectively   These values represent 47 8 and 50 9 per cent, respectively, of the total base administered   The average ratios between the bound and free forms were 7 2 during morphine and 8 5 during heroin administration

DISCUSSION   It is commonly believed that heroin hydrochloride, kept in a dry form, is not stable over a long period of time   One sample had a specific rotation of −158 0°, when it was prepared   Five months later the rotation had dropped to −153 7°   Another sample gave a rotation of −156 1° when it was prepared, and four years later was −147 4°   It is certain that the first sample contained no water of crystallization   Just how much hydrolysis has taken place in the sample with a 4 3° decrease in rotation is not certain, but it is probably not more than 4 per cent

From the present study it seems reasonable to conclude that heroin hydro-chloride is excreted as morphine. Since heroin hydrochloride added to urine hydrolyzes very readily by the present technique, it is not probable that the body would excrete unchanged heroin. If the excreted uncombined form is morphine, the bound form is also likely to be morphine. Acid hydrolysis of the conjugate would liberate morphine regardless of the presence of an acetyl group It has been shown (10) that conjugation does not take place when both the phenolic hydroxyl and the secondary alcoholic hydroxyl groups are methylated, therefore unchanged heroin could not be expected to form a conjugate. Monoacetyl derivatives could form conjugates, but since no acetyl derivatives in the uncom-bined form were found in the urine, it is not probable that these would be present in a conjugated form.

In this study the relative physical dependence satisfying power of heroin hydro-chloride was about twice that of morphine sulfate. This does not imply that heroin is twice as addictive as morphine, but that approximately one-half as much heroin as morphine is needed to satisfy physical dependence in a morphine addict. Even though the dosage ratio of morphine to heroin in this study was about 2 to 1, the average daily per cent of free and bound morphine base in urine was approximately the same for both drugs.

SUMMARY

In alkaline solution (0.5 M Na_2CO_3 at 26°C.) heroin hydrolyzes rapidly. Approximately 58 per cent of the total sample hydrolyzed (calculated as mor-phine base) in 2 minutes, and 88 per cent in 10 minutes. Calculations made from results of these tests indicate that at least 96 per cent of the sample tested was diacetylmorphine hydrochloride.

As to the fate of heroin in the human body, evidence is presented indicating that all of the drug is hydrolyzed completely. It is not possible to extract heroin hydrochloride added to urine without causing some hydrolysis.

Physical dependence on morphine was satisfied by heroin hydrochloride. The ratio of physical dependence satisfying doses of morphine sulfate and heroin hydrochloride was 2 to 1. On this dosage ratio approximately 7 per cent of the administered drugs were excreted in free form and 50 per cent in bound form.

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EFFECT OF REPEATED APPLICATIONS OF SUBLETHAL CONCENTRATIONS OF GERMICIDES ON LIVING EMBRYONIC TISSUE FRAGMENTS

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Some germicides, notably the mercury and the silver compounds, are known to accumulate in the living organism when repeatedly administered while others, such as the halogen compounds and the phenols, do not. It was considered, therefore, of great interest and importance to see if tissue fragments could be used in a similar manner to demonstrate a cumulative action

METHOD Chick heart tissue fragments from 0 5 to 1 mm in diameter were embedded in rabbit plasma contained in 35 mm Carrel flasks (0 3 cc plasma, 0 7 cc Tyrode's solution, 0 3 cc embryonic extract, 0 2 cc tissue suspension containing about 12 fragments) After the plasma had coagulated, 1 cc of embryonic extract was added as a nutrient (total vol ume, 2 5 cc) The cotton stoppers were replaced with sterile paraffined corks to prevent the loss of carbon dioxide and the flasks incubated at 37°C

The flasks were prepared either at 10 A M or 2 P M , then treated with germicide the following morning at 10 A M and every 12 hours thereafter Four flasks were selected for each germicide and 4 for the control The embryonic extract was aspirated from the flasks and the selected fragments photographed Then the flasks were each treated with 1 cc of the appropriate germicidal dilution The dilutions selected were approximately one fif teenth of the concentrations required to kill the tissue fragments in 10 minutes at 37°C in the absence of organic matter They were as follows Iodine, 1 30 000, Hexylresorcinol, 1 60,000, mercuric chloride, 1 150 000, Metaphen, 1 350,000, Merthiolate, 1 440 000, silver nitrate, 1 15,000, silver protein solution, U S P , 1 3,000 The solutions were freshly pre pared before each treatment All dilutions were made in 0 85% sodium chloride solution except silver nitrate, which was dissolved in distilled water The flasks with germicide were incubated at 37°C for 10 minutes At the end of the exposure period the germicidal dilutions were aspirated from the flasks, 1 cc of embryonic extract added again stoppered with paraffined corks, and returned to the 37°C incubator This procedure was repeated twice daily at 10 A M and at 10 P M for 12 days

At the end of the tenth day transplants were prepared from several fragments in each set to determine if the tissues were still living The transplants selected were not necessarily prepared from the fragments previously photographed

IODINE Large doses of iodine are required to produce death in man and animals, which indicates that it is not highly toxic After absorption the iodine ion is entirely extracellular, except for its penetration into red blood cells The element is not a cumulative poison It is readily excreted by the kidneys, most of it being eliminated within 24 hours after administration There is very little retention of iodide and that which remains is probably in organic combination

A typical set of results is shown in Fig 1 It may be seen that the tissue fragments treated with a sublethal concentration of iodine for 10 days (20 treat ments) grew in a normal manner like the controls (Fig 2) The fibroblasts appeared normal in appearance and in the rate of growth throughout the experi

mental period. There was apparently no cumulation of iodine in the fragments. A transplant from a fragment previously treated for 10 days continued to reproduce in a normal manner indicating that the germicide produced no appreciable effect on the tissue.
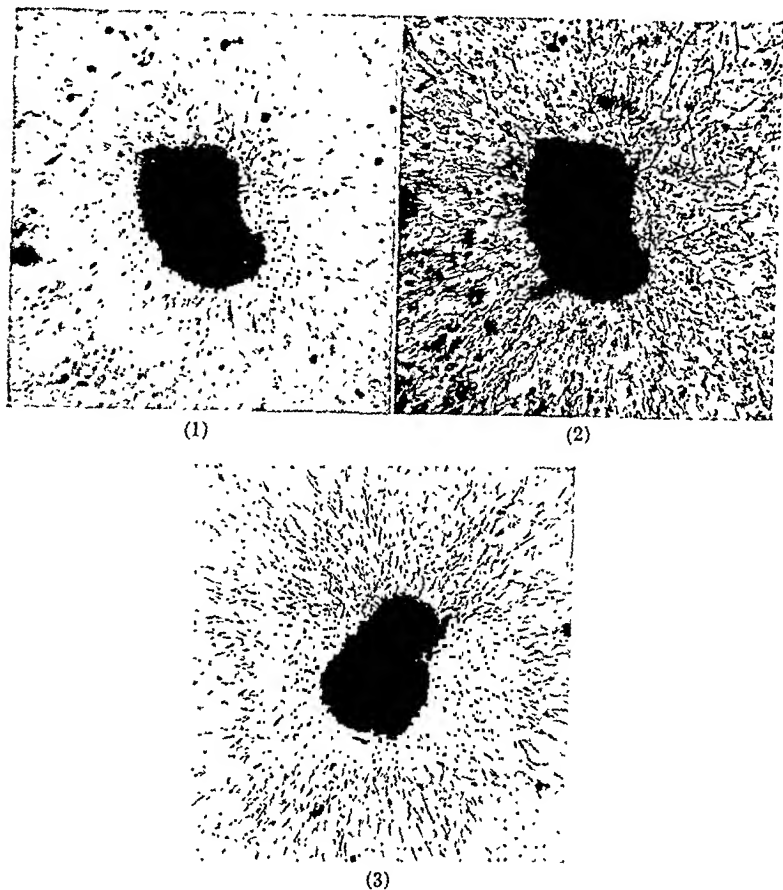
FIG. 1. TISSUE FRAGMENTS TREATED WITH IODINE

(1) 24 hours old, treated 2 times; (2) 216 hours old, treated 18 times; (3) transplant from fragment treated for 240 hours (from another set).

The flasks treated with iodine contained a number of fragments still pulsating in a vigorous manner after 12 days. On the other hand the control flasks showed no pulsating fragments beyond 96 hours.

HEXYLRESORCINOL. Hexylresorcinol is not a cumulative drug and may be repeatedly administered without serious effects. After oral administration about one-third of the drug is absorbed. It is excreted by the kidneys and

appears in the urine largely in a conjugated form. The unabsorbed portion is eliminated unchanged in the feces.

The results of the action of Hexylresorcinol on living tissue fragments are shown in Fig. 3. The repeated application of Hexylresorcinol over a period of 12 days did not affect the character or the rate of growth of the fibroblasts. The
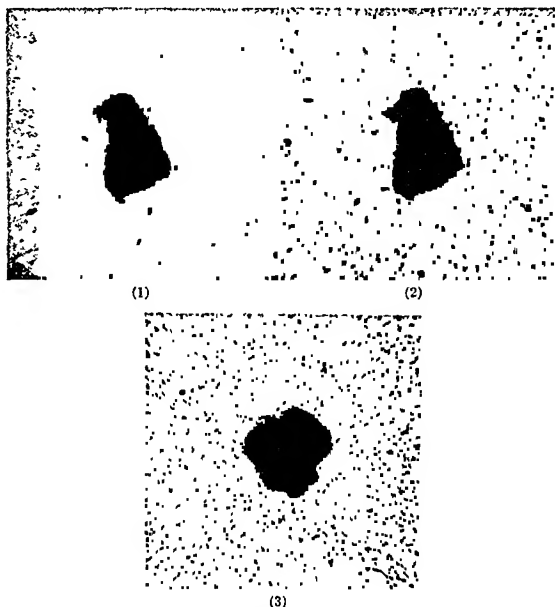
FIG. 2. CONTROL TISSUE FRAGMENTS, NOT TREATED
(1) 24 hours old; (2) 216 hours old, (3) transplant from fragment 240 hours old (from another set).

tissues appeared normal in every respect. A transplant from a fragment previously treated for 10 days continued to grow in a normal manner.

MERCURIALS The mercury ion has but one pharmacological action (1): it is a protoplasmic poison because it readily precipitates proteins. All mercury compounds are potential sources of the ion. The toxicity of mercury compounds

depends upon the ease with which the ion is liberated. Since mercuric chloride ionizes more than any other compound of mercury it is also the most toxic.

The organic mercury compounds are less toxic than the inorganic ones. The mechanism of their action is not clearly understood. They are not as highly ionized as the inorganic compounds, yet are much more active. However, it is

FIG. 3. TISSUE FRAGMENTS TREATED WITH HEXYLRESORCINOL
(1) 24 hours old, treated 2 times; (2) 216 hours old, treated 18 times, (3) transplant from fragment treated for 240 hours.

generally agreed that part of their toxicity is also due to release of low concentrations of mercury ions.

When mercury enters the circulation it is rapidly taken up by the tissues (2, 3). The continued administration of mercury over a period of time may result in chronic poisoning (4).

Mercuric chloride. The effect of repeated applications of a sublethal concentration of mercuric chloride to tissue fragments is given in Fig. 4. The fragments showed normal proliferation only during the first 72 hours (6 treatments). After that the fragments showed no further growth and the fibroblasts rapidly degenerated. Apparently mercury accumulated in the tissue fragments until
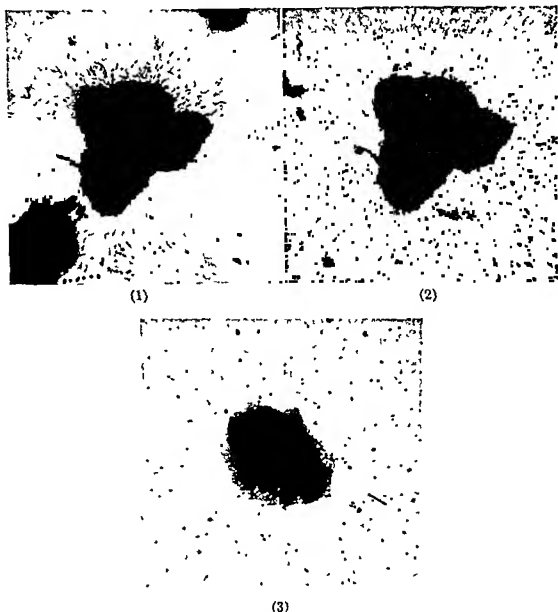
FIG. 4. TISSUE FRAGMENTS TREATED WITH MERCURIC CHLORIDE
(1) 24 hours old, treated 2 times; (2) 216 hours old, treated 18 times; (3) transplant from fragment treated for 120 hours (from another set)

death was produced. This was further shown by the fact that transplants from fragments previously treated for only 5 days failed to grow. The tissue fragments ceased to pulsate after 48 hours, another indication of the damaging effect of the mercury ion.

Merthiolate and Metaphen. Entirely different results were obtained from the

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(1)   (2)

(3)

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When mercury enters the circulation it is rapidly taken up by the tissues (2, 3). The continued administration of mercury over a period of time may result in chronic poisoning (4).

SILVER COMPOUNDS. Silver preparations are employed largely for their local actions. They are used for this purpose either in the form of inorganic salts or in organic combinations with proteins. The silver ion precipitates the proteins of tissues. There are two phases in its action: *(1)* Proteins are quickly precipitated in the presence of the silver ion of highly ionizable inorganic salts;
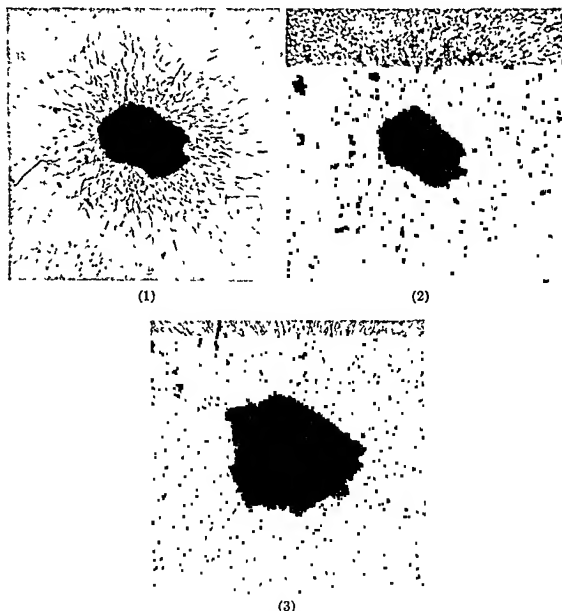
FIG. 6 TISSUE FRAGMENTS TREATED WITH METAPHEN
(1) 24 hours old, treated 2 times, (2) 216 hours old, treated 18 times; (3) transplant from fragment treated for 240 hours

(2) this is followed by a more sustained action due to the slow release of small quantities of ionic silver from the silver protein compound. The colloidal silver preparations behave according to the second type of action.

Silver is absorbed into the system, being found largely in the connective tissue and corium of the skin. Since silver is rapidly precipitated in the presence of proteins, it is not possible to attain appreciable concentrations of the element in

the circulation.   Some silver is absorbed from mucous membranes and this is largely retained.

Silver nitrate.   The results of the action of silver nitrate on embryonic tissue fragments are shown in Fig. 7.   It may be seen that the salt produced considerable damage to the fibroblasts after 72 hours.   The fibroblasts quickly degen-
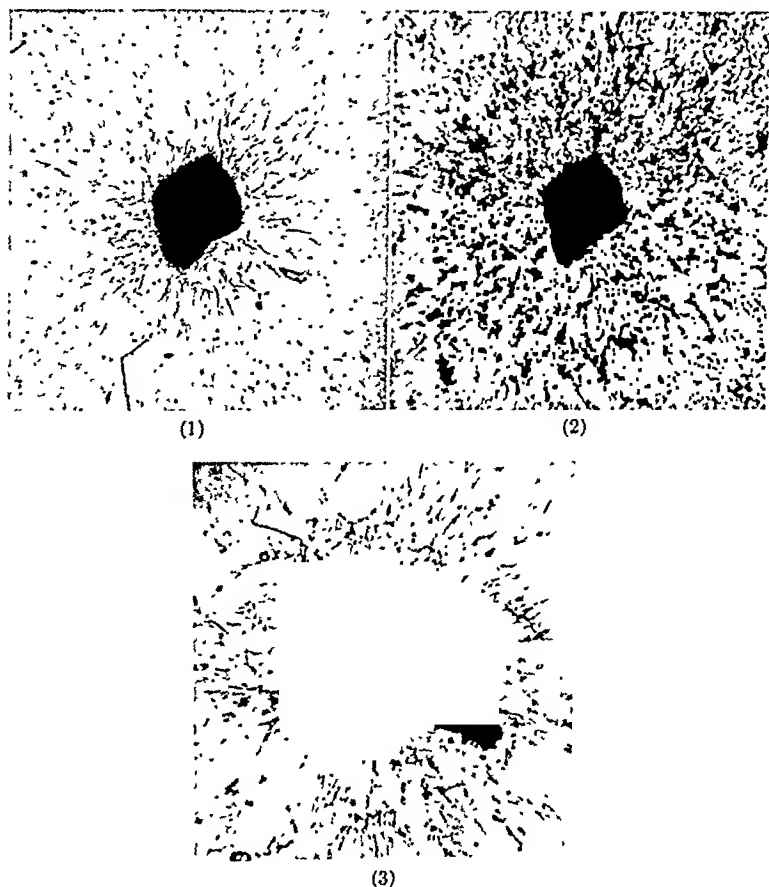
FIG. 7. TISSUE FRAGMENTS TREATED WITH SILVER NITRATE
(1) 24 hours old, treated 2 times; (2) 216 hours old, treated 18 times; (3) transplant from fragment treated for 240 hours.

erated and separated from the tissue fragments.   After the tenth day most of the fragments were completely separated from the fibroblasts.

The tissue fragments pulsated for 10 days, then ceased.   However, transplants prepared from fragments which had ceased to pulsate continued to multiply but at a slower rate than the controls.

The slower action of silver nitrate compared to mercuric chloride might be explained on the fact that silver is rapidly precipitated in the presence of chloride ions whereas mercury is not. Tyrode's solution, used in preparing the embryonic extract and in diluting the plasma, contains a relatively high concentration of

(1)

(2)

(3)
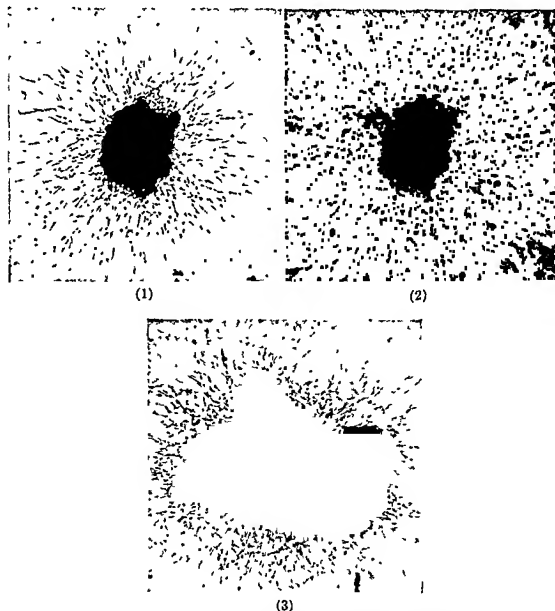
FIG. 8. TISSUE FRAGMENTS TREATED WITH SILVER PROTEIN STRONG

(1) 24 hours old, treated 2 times, (2) 216 hours old, treated 18 times; (3) transplant from fragment treated for 240 hours

chloride ions. Because of this fact most of the silver was prevented from reaching the tissue fragments.

Silver Protein Strong. The compound produced extensive injury on the fibroblasts (Fig 8) but not as much as in the case of silver nitrate. After the tenth day some of the tissue fragments were separated from the fibroblasts but not as many as were observed in the flasks treated with silver nitrate.

The tissue fragments pulsated vigorously throughout the entire experimental period of 12 days. Transplants prepared from fragments treated for 10 days continued to proliferate at a normal rate.

SUMMARY

The results reported show that tissue fragments cultivated *in vitro* may be successfully employed to demonstrate the presence of a cumulative action in germicidal compounds or preparations.

Iodine and Hexylresorcinol did not exhibit an appreciable cumulative action on proliferating tissue when applied twice daily for 12 days.

Under the same conditions mercuric chloride exhibited a pronounced cumulative action after 72 hours resulting in the death of the tissue fragments.

The organic mercurials Merthiolate and Metaphen produced considerable damage to the fibroblasts but did not exhibit an appreciable cumulative effect on the tissue fragments. The fragments remained vigorous throughout the experimental period.

Silver nitrate produced considerable damage on the fibroblasts and a slow cumulation of silver in the tissue fragments.

The organic silver protein strong also produced considerable damage on the fibroblasts but the tissue fragments remained vigorous throughout the experimental period, indicating that the cumulation of silver was negligible.

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THE METABOLISM OF PARALDEHYDE

I. The Determination of Paraldehyde in Tissues, Blood and Expired Air[1]

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DETERMINATION OF PARALDEHYDE   In order to be able to determine the small amounts of paraldehyde present in the blood, tissues and expired air of mice after the drug was administered, the following methods have been developed They have been employed in an investigation of the metabolism of paraldehyde (1)

1 *The determination of paraldehyde in blood*   The reaction between p hydroxydiphenyl and acetaldehyde in concentrated sulfuric acid was first described by Legriwe (2)   It was adapted by Miller and Muntz (3) to the determination of lactic acid, and has been used for the determination of this substance by Stone (4), Koenemann (5) and Barker and Summerson (6)   Block and Bolling (7) have used the reaction for the determination of threonine, alanine, lactic acid and alcohol, and Barker (8) has employed it for the estimation of the small amount of acetaldehyde normally present in blood   The following adaptation to the determination of paraldehyde in blood has proved satisfactory

Reagents   Zinc Sulfate, 10 per cent, Sodium Hydroxide, 2 N , p-Hydroxydiphenyl, Sulfuric Acid, concentrated, Paraldehyde, standard solutions prepared by dilution of U S P   paraldehyde and checked by placing an amount expected to contain 1 mgm in the tissue paraldehyde apparatus and determining the actual content as described below

PROCEDURE   A 0 20 cc sample of blood is pipetted into a centrifuge tube containing 1 80 cc of distilled water the tube tightly stoppered, shaken and allowed to stand for a few minutes   Then 0 50 cc of ZnSO₄ solution is added with shaking followed by 0 50 cc of 2 N NaOH, the tube is shaken, allowed to stand for about thirty minutes and centrifuged within two hours

One cc of the supernatant fluid from each blood sample is placed in a test tube, a series of standards is prepared by placing in other test tubes 1 cc of standard solutions containing amounts of paraldehyde corresponding to those anticipated in the unknowns   All tubes are stoppered and kept in an ice bath to minimize volatilization of the paraldehyde   To each tube 10 cc of ice cold concentrated sulfuric acid are added while the tubes are being

shaken in the ice bath. The first additions must be made in small fractions, each addition followed by shaking, in order to prevent the temperature from rising, since at temperatures much above room temperature, lactic acid is oxidized to acetaldehyde. The same batch of cold acid must be used for all tubes of one run, for variations in the water content of acid greatly affect the color developed. Then to each tube 100 mgm. of p-hydroxydiphenyl are added. The tubes are shaken thoroughly and allowed to stand for at least an hour for full color development. The contents are then poured into colorimeter tubes, and, after the solid reagent has come to the surface, the unknowns are compared with the standards.

The relation between paraldehyde concentration and color density follows Beer's Law to quantities of paraldehyde up to 100 mgm. per cent.

Table 1 illustrates the recovery of paraldehyde added to blood to make the specified concentrations.

TABLE 1

Recovery of paraldehyde added to blood

PARALDEHYDE ADDED	PARALDEHYDE RECOVERED	PER CENT RECOVERY	PARALDEHYDE ADDED	PARALDEHYDE RECOVERED	PER CENT RECOVERY
mgm. per cent	*mgm. per cent*		*mgm. per cent*	*mgm. per cent*	
5.0	5.2	104	30.0	30.0	100
	4.9	98		31.0	103
	4.7	94		29.3	98
10.0	10.0	100	40.0	40.6	102
	10.6	106		41.0	103
	10.2	102		40.2	101
20.0	20.7	104	50.0	48.4	97
	20.2	101		49.9	99
	20.3	102		49.5	99

DISCUSSION. There is a small blank value for blood for which it has been possible to compensate by subtracting from the determined value the value of the average of a number of determinations of the blank from control animals. In the case of mouse blood, ten such determinations gave a mean of 4.5 mgm. per cent and a variation of plus or minus 0.5 mgm. per cent. This blank is probably due to the acetaldehyde normally present in blood (8). This point was not investigated in the present study.

The layer of solid reagent above the colored solution is troublesome. In the Klett-Summerson (9) colorimeter used in the present study, and others having a horizontal light beam traversing a perpendicularly placed tube, it is only necessary to make the layer of acid deep enough for the solid to lie above the light beam. It is not practicable to employ this method when such a colorimeter is not available. In the method for lactic acid mentioned above, the solid is dissolved by heating in a boiling water bath. This cannot be done in the present method, since the lactic acid present would be oxidized to acetaldehyde at this temperature.

Previous experimenters (3–8) have found it necessary to weigh carefully the

p hydroxy diphenyl added to each tube   In experiments in connection with the present study, it was found that as long as an excess is added to each tube, the final color is independent of the quantity added, but that the rate of color development is greatly affected   Thus in a typical experiment, color development was complete in a tube to which 200 mgm were added in ten minutes, but was not complete for over three hours in one in which a bare excess was added   This seems to be due to the very meagre solubility of the reagent in concentrated sulfuric acid, approximate determinations indicate a solubility of the order of 1 mgm per liter   Since most workers have added small quantities and allowed the reaction to proceed for a short time, the dependence of color density on reagent added seems to be explained   The majority of the color development seems to occur on the surfaces of the solid   This is indicated by the fact that in an undisturbed tube, the color develops in a narrow band adjacent to the layer of solid, and can be seen to diffuse downward   Moreover, coarse crystals are seen to be distinctly colored at their edges   Finally, in two tubes prepared exactly alike, except that in one the reagent was added in the form of coarse crystals weighing about 10 mgm each and in the other as a very fine powder, color development was complete in the second in about fifteen minutes, and in the first only after more than two hours   During this time, the tubes were frequently shaken, in order to distribute the solid throughout the solution

2 *The determination of paraldehyde in tissues and expired air*   Attempts to apply the method described above to tissues were unsuccessful   It was found that it is very difficult to extract paraldehyde completely from tissues and that it can only be done with inconveniently large volumes of extracting solution   The following procedure, based on the method for lactic acid determination of Clausen (10) and Friedemann, Cotonio and Shaffer (11) was employed   The principle is depolymerization of the paraldehyde to acetaldehyde by hot dilute sulfuric acid, aeration of the acetaldehyde into a solution of sodium bisulfite and iodometric titration of the bound bisulfite

Nitzescu, Georgescu and Timus (12) attempted a similar determination of tissue paraldehyde using trichloracetic acid extracts   As mentioned, the present experiments indicated that such extractions are not likely to be quantitative   The apparently adequate recoveries of these authors are to be explained by their use of the factor for converting cc of 0 001 N iodine consumed in the titration of the bisulfite to mgm of paraldehyde of 0 044 mgm per cc , rather than the correct factor of 0 022 mgm per cc

The apparatus used is essentially that for the lactic acid determination (11), except that no provision is made for adding permanganate   The chopped tissue is placed in the boiling flask which has a capacity of 500 cc , and 200 cc of 20 per cent sulfuric acid are added   The mixture is boiled while a stream of air is drawn through it   As the tissue dissolves, the paraldehyde is depolymerized and transported into the absorption tower, where it is combined with the sodium bisulfite   It was found by test that when the tissue has dissolved, yielding a homogeneous brown solution, all the paraldehyde has been carried into the bisulfite   In the case of the mouse carcass, this required about an hour   Then the contents of the tower are titrated as usual with standard iodine

The tissues of the mouse show a blank, which, in the case of the carcass, amounts to about 0.3 mgm. This has been compensated for by subtracting from the value determined on each experimental animal the blank determined on a control mouse of the same weight simultaneously. Table 2 exhibits the recoveries obtained of paraldehyde injected intravenously into mice. The mice

TABLE 2

Recovery of intravenously injected paraldehyde

AMOUNT INJECTED	AMOUNT RECOVERED	PER CENT RECOVERY
mgm.	*mgm.*	
0.20	0.23	113
0.50	0.48	96
1.00	0.93	93
5.00	4.93	98
15.0	14.4	96
25.0	22.1	88
50.0	49.9	99

TABLE 3

Recovery of paraldehyde in the expired air apparatus

AMOUNT ADDED	AMOUNT RECOVERED	PER CENT RECOVERY	AMOUNT ADDED	AMOUNT RECOVERED	PER CENT RECOVERY
mgm.	*mgm.*		*mgm.*	*mgm.*	
0.010	0.0120	120	0.500	0.500	100
	0.0100	100		0.491	98
	0.0105	105		0.485	96
	0.0096	96		0.508	102
	0.0102	102		0.502	101
0.050	0.047	94	1.00	1.03	103
	0.049	98		1.01	101
	0.051	102		0.99	99
	0.048	96			
	0.050	100			
0.100	0.110	110	5.00	4.79	96
	0.097	97		4.89	98
	0.104	104		5.03	101
	0.095	95			
	0.101	101			

were injected, killed immediately and the determinations begun as rapidly as possible.

For the determination of paraldehyde in expired air, essentially the same procedure was used as that employed by Nitzescu *et al.* (12) and by Defandorf (13). The apparatus for tissue paraldehyde determination was modified by placing the boiling flask, containing 200 cc. of sulfuric acid, in a boiling water bath and con-

necting the air inlet tube to a jar in which the mouse was placed   A stream of air is drawn through the animal jar, the expired paraldehyde transported into the flask containing the hot sulfuric acid, where it was depolymerized to acetaldehyde, which is combined in the bisulfite absorption tube   At intervals of 15 or 30 minutes, the contents of the absorption tower are removed, more bisulfite added, and the bound bisulfite in each sample titrated with standard iodine as in the usual procedure.

Table 3 exhibits the recovery of paraldehyde obtained from solutions of the substance placed on watch glasses in the animal chamber.

It should be noted that the method employed for the determination of expired air paraldehyde by Levine and Bodansky (14) might have been employed in these determinations   There is no difference in the suitability of their method as compared with that employed in this investigation

<div align="center">SUMMARY</div>

Methods for the determination of paraldehyde in blood, tissues and expired air are described.   The determination in blood is accomplished by means of the color reaction between paraldehyde and p-hydroxydiphenyl in sulfuric acid, the estimations in tissues and expired air are performed by depolymerization of the paraldehyde to acetaldehyde in hot dilute sulfuric acid and absorption of the liberated acetaldehyde in sodium bisulfite solution, followed by iodometric titration.   The methods have an accuracy of about 5 per cent

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THE METABOLISM OF PARALDEHYDE. II[1]

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Information regarding the metabolism of paraldehyde is incomplete, and the few published studies are contradictory. Conn (1) was unable to isolate any products of its metabolism from the urine of dogs. La Rue (2), also using dogs, after a dose of 1.8 gm. per kgm. by stomach found the blood free of paraldehyde at the 14th hour and only traces in the urine at the 24th hour. Nitzescu, Georgescu and Timus (3, 4) reported that rats excreted through the lungs 100 per cent of an intravenous dose of 1 gm. per kgm. in 10 hours, whereas rabbits given the same dose intravenously eliminated 22.8 per cent in the expired air in 10 hours. Defandorf (5) following the excretion of paraldehyde given to dogs by rectum, found relatively small amounts excreted in the urine or by way of the lungs. Levine, Gilbert and Bodansky (6, 7) in recent studies have shown that in dogs given narcotic doses by stomach tube, up to 28 per cent was excreted by way of the lungs, and much smaller amounts, less than 3 per cent, by the kidneys. In the same animals, after liver damage had been produced by chloroform anesthesia, more paraldehyde was excreted from the lungs, blood concentrations fell more slowly, and the hypnotic action was prolonged. Somewhat more of the drug, about 5 per cent, was excreted in the urine. These authors believed that the liver plays a dominant rôle in the destruction of paraldehyde. When the functional capacity of the liver was reduced, the slower rate of destruction resulted in the maintenance of higher blood levels for longer periods, with increased pulmonary and renal excretion. With the exception of the single report of Nitzescu, Georgescu and Timus (3) that the rat excretes 100 per cent of an injected dose, all workers are agreed that most of the paraldehyde is destroyed. No recent experiments designed to reveal the fate of this unexcreted fraction have been found. The present study was undertaken to obtain more complete information on the rate of excretion at different dosage levels, both in normal animals and after liver injury, and also, if possible, to determine what happens to that fraction of the paraldehyde which is not excreted.

EXPERIMENTAL. Since it was desired to determine not only the rate of pulmonary excretion of paraldehyde, but also blood concentration and the amounts remaining in the

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body after different intervals and doses, it was necessary to work with small animals These were a pure bred strain of albino Swiss mice, raised in the laboratory, fed on a diet of Purina dog chow or bread and milk, with occasional green vegetables added   When it was desired to produce liver injury, carbon tetrachloride in doses of 1 cc per kgm dissolved in olive oil to make a 20 per cent solution was injected subcutaneously, usually 24 hours before the paraldehyde was to be given   No report on the pathological histology of the liver of the mouse after carbon tetrachloride poisoning has come to the authors' attention It may be stated that the findings obtained on a series of 24 such livers examined did not differ essentially from those described by Cameron and Karunaratne (8) for the rat

The methods for determining paraldehyde in the expired air, blood and tissues of the mice are described in another communication (9)   The urinary excretion of paraldehyde
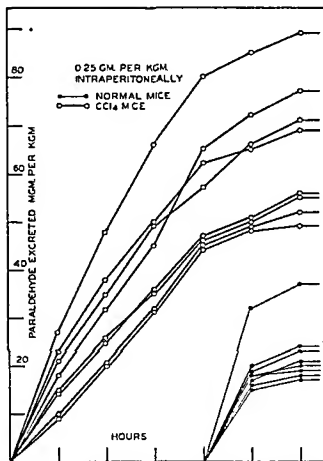
FIG 1 Pulmonary excretion of paraldehyde after a dose of 0 25 gm per kgm intra-peritoneally   Closed circles represent normal mice open circles carbon tetrachloride mice

was not examined in this study   Doses of paraldehyde given were as follows orally, 1 0, 0 5, 0 25 0 1 and 0 05 gm per kgm , intraperitoneally , 0 5, 0 25, 0 1, 0 05 and 0 025 gm per kgm   These represent the range from the largest tolerated dose to the smallest after which excretion of paraldehyde through the lungs could be detected   Determinations were made at 15 to 30 minute intervals until no further excretion could be detected   Data were obtained in all cases on 8 normal and 8 carbon tetrachloride mice with the exception of the largest oral dose tolerated by the carbon tetrachloride mice, 0 5 gm per kgm , which only 3 out of 12 mice survived, and of the oral dose of 1 gm per kgm to normal mice, in which 16 animals were used

RESULTS   The results obtained at the different dosage levels were strikingly regular and consistent   In figure 1 are given the detailed data obtained from a

single experiment in which the pulmonary excretion of paraldehyde was determined after administration of 0.25 gm. per kgm. intraperitoneally to 8 normal and 8 carbon tetrachloride mice. The curves were constructed by adding the amount of paraldehyde excreted in each period to the total already excreted. It is obvious that excretion begins promptly, and gradually falls to zero. In the case of the normal mice, no more paraldehyde was eliminated after 2 hours. The mean excretion in the group of normal mice was 20.3 mgm. per kgm. which
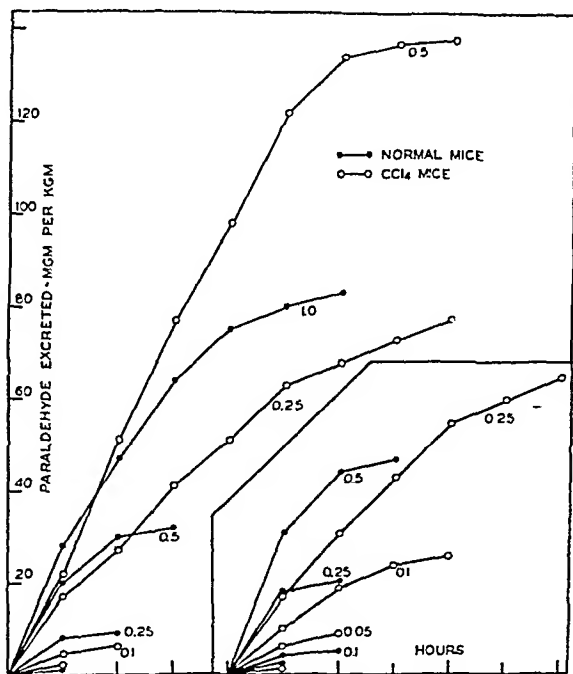
FIGS. 2 AND 3. Curves representing pulmonary excretion of paraldehyde after various doses to normal and carbon tetrachloride mice. Figure 2 gives data for oral administration of paraldehyde, figure 3 for intraperitoneal administration. Closed circles represent normal mice, open circles carbon tetrachloride mice. The numbers near each curve are the doses administered to the group of mice. In figure 2, the two lowest curves, for which the doses are unspecified, are a dose of 0.05 gm. per kgm. to carbon tetrachloride mice and of 0.1 gm. per kgm. to normal mice. The two lowest, unlabeled curves in figure 3 are for doses of 0.05 gm. per kgm. to normal mice and 0.025 gm. per kgm. to carbon tetrachloride mice.

is approximately 8 per cent of the amount administered. In the carbon tetrachloride group, excretion ceased only after 6 hours, and the mean excretion for the group was 65.3 mgm. per kgm., which is approximately 26 per cent. The results of all other experiments were similar to this, differing only in the time over which excretion occurred and the amounts excreted. The spread between the excretion of individual members of each group was of the same order as that depicted in figure 1. For this reason, *only the mean excretion for the various*

groups are presented in figures 2 and 3. Figure 2 shows the results after oral administration and figure 3 after intraperitoneal. It should be noted that no curve is shown for carbon tetrachloride mice corresponding to the largest dose shown for the normal mice for each route of administration, because each of these doses was fatal in the carbon tetrachloride animals. At the other extreme, for

TABLE 1

Excretion and destruction of paraldehyde

DOSE	PARALDEHYDE EXCRETED	TIME OF EXCRETION	RATE OF EXCRETION 1ST HOUR	MEAN RATE OF EXCRETION	MEAN RATE OF DESTRUCTION
Paraldehyde given orally, normal mice					
mgm per kgm	*mgm per kgm*	*hours*			
1000	83 4	6	28.4	13.9	152
500	32 3	3	19 8	10 8	156
250	9.0	2	8 5	4.5	121
100	1.4	1	1.4	1.4	99
Paraldehyde given orally, CCl₄ mice					
500	138.3	7	22.2	19.8	52
250	75 3	7	17 3	10.8	25
100	4.9	2	4.7	2.5	48
50	1.5	1	1 5	1.5	49
Paraldehyde given intraperitoneally, normal mice					
500	47.3	3	31.2	15 8	151
250	20.3	2	18.1	10 2	115
100	4.8	1 5	4.7	3 2	64
50	1 8	1	1.8	1.8	48
Paraldehyde given intraperitoneally, CCl₄ mice					
250	65 3	6	16 9	10.9	31
100	26 0	3	10 0	8.6	25
50	7 8	2	6 8	3.9	21
25	1 6	1	1.6	1 6	23

All rates are given in mgm per kgm per hour. The data in each group are the averages for 8 mice, except in the dose of 1 gm per kgm, where 16 mice were used, and in the dose of 0 5 gm per kgm orally to CCl₄ mice, where only 3 of 12 mice survived.

the smallest dose shown for the carbon tetrachloride mice, there is no corresponding curve for the normal mice, since no excretion could be detected in these.

Table 1 was prepared from the combined data in the following manner. The average amount of paraldehyde excreted through the lungs by each group of mice, divided by the number of hours over which excretion occurred, gives the value called "mean rate of excretion". The average amount of paraldehyde excreted in the first hour by each group of mice is called the "rate of excretion first hour". The "mean rate of destruction" of paraldehyde was calculated by subtracting

the total amount of paraldehyde excreted from the amount administered, and dividing by the number of hours over which·excretion occurred, on the assumption that when excretion had ceased, destruction of the drug was practically complete.

The following conclusions seem to be justified by the data summarized in the table. In any particular series of experiments, the rate of excretion during the first hour and over the whole period, as well as the time of excretion and the quantity excreted, are functions of the dose. In every case, however, the quantity excreted is less than the quantity destroyed.[4]

In the series of normal animals, the rate of destruction is a function of the dose administered, except at the higher levels. In the carbon tetrachloride series, however, the rate appears to be approximately constant, but different for the different routes of administration. The authors can offer no explanation for the discordant values in the case of the dose of 0.25 gm. per kgm. given orally to carbon tetrachloride mice. Excretion for this group continued much longer than is to be predicted on the basis of experience with the other groups of mice, and the rate of destruction is consequently much lower.

A possible explanation of the constant rates of destruction at high doses in the normal mice and at all doses in the carbon tetrachloride series consists in the assumption that there is a maximum rate of destruction and that increases in dose above the maximum capacity do not consequently cause increases in rate of destruction. It may be assumed that carbon tetrachloride causes interference in some way with this destructive mechanism, as a possible explanation of the lower and constant rates of destruction of paraldehyde in these animals. The findings with respect to carbon tetrachloride poisoning are taken by the authors to indicate that the liver is the site of an important step in the processes by which paraldehyde is destroyed, as was suggested by Levine et al. (6). There are no data to indicate that the liver is the exclusive site of this process.

Three other series of experiments have been performed in connection with the pulmonary excretion of paraldehyde. In the first of these the doses of carbon tetrachloride and paraldehyde were constant at 1 cc. per kgm. subcutaneously and 0.25 gm. per kgm. intraperitoneally, but the interval between the administration of carbon tetrachloride and paraldehyde was varied, the intervals studied being 6, 12, 24, 36, 48, 72 and 96 hours. It was found that the increase in excretion of paraldehyde was greatest at 24 hours and had returned to normal by 72 hours. This corresponds with the time of greatest liver damage, as seen in histological examination.

[4] Nitzescu, et al. (3) stated that the rat excretes 100 percent of an intravenous dose of paraldehyde through the lungs. In view of the fact that this finding is inconsistent with all other data, it seemed appropriate to reexamine these findings very briefly. Two rats were found to excrete 13 and 15 percent of a dose of 0.25 gm. per kgm. of paraldehyde intraperitoneally. The data of Nitzescu et al. may be at least partially explained by the fact that they used the wrong factor for converting cc. of 0.001 N. iodine used in titrating the bound bisulfite to mgm. of paraldehyde, employing the factor of 0 044 mgm per cc., rather than the correct one of 0.022 mgm. per cc. Hence, their results indicate an excretion of not more than 50 percent of the dose given.

In the second series of experiments, the dose of paraldehyde was kept constant, at 0 25 gm per kgm intraperitoneally, but the dose of carbon tetrachloride was varied from 0 1 to 10 cc per kgm The interval between the times of administration of the carbon tetrachloride and paraldehyde was 24 hours It was found, as expected, that the quantity of paraldehyde excreted increased as the dose of carbon tetrachloride was increased It may be noted that the authors have not found even the largest of these doses of carbon tetrachloride to be fatal in this colony of mice, when administered as a single dose, subcutaneously, in 20 per cent solution in olive oil
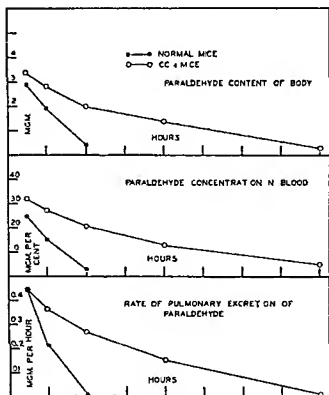
F c 4 D t the ll d c f th l t d ll h d t

The third series of experiments was to check on the blood concentration and carcass content of paraldehyde It is reasonable to assume that the increased rate and duration of excretion of paraldehyde after carbon tetrachloride poisoning indicate that the concentration in the blood and quantity still present in the body are greater than in the normal at any particular time after the administration of the paraldehyde It was felt worthwhile actually to make these determinations In this experiment the dose of paraldehyde was 0 25 gm per kgm intraperitoneally and of carbon tetrachloride 1 cc per kgm In each series, normal and carbon tetrachloride, the rate of excretion, blood concentration and body content fell in parallel fashion, and after carbon tetrachloride, all three fell much more slowly (figure 4) The observation of Levine, Gilbert and Bodansky

(6, 7) on blood content and pulmonary excretion were thus confirmed, as was their observation that the narcotic action of the drug is much prolonged. Thus, the normal mice responded to vigorous stimulation about forty-five minutes after the drug was administered, whereas the carbon tetrachloride mice did not do so for about two hours.

It was a part of the purpose of these studies to investigate the path of destruction of paraldehyde. It appeared at the outset that the most reasonable hypothesis is that paraldehyde is depolymerized to acetaldehyde which is the oxidized, probably to acetic acid, and eventually to carbon dioxide and water.

Accordingly, an attempt was made to isolate acetaldehyde from the expired air of animals to which paraldehyde had been administered. The animals, after a dose of 0.25 gm. per kgm. intraperitoneally, were placed in the apparatus for the determination of paraldehyde in the expired air, which was modified for the purposes of this experiment by inserting a bisulfite absorption tower between the animal chamber and the sulfuric acid flask (9). No excretion of acetaldehyde was detected. It was found by experiments with mixtures of acetaldehyde and paraldehyde placed in the animal chamber, that if the animals had excreted as acetaldehyde 0.1 per cent of the paraldehyde administered, it could have been detected.

This finding did not necessarily invalidate the working hypothesis mentioned above, if acetaldehyde were metabolized by the mice at a rate greatly in excess of that for paraldehyde. The pulmonary excretion of administered acetaldehyde was therefore investigated, using the same equipment as that previously employed for the paraldehyde studies. The drug, freshly prepared each day, was administered in aqueous solution in doses of 0.5 and 0.25 gm. per kgm. orally and 0.25 and 0.1 gm. per kgm. intraperitoneally. The first of these doses in each case represents the largest tolerated and the second the smallest after which any excretion could be detected. Each dose was administered to 8 normal and 8 carbon tetrachloride mice. Excretion was complete in every case in 30 minutes, and only small quantities, about 2.5 per cent of the administered dose, were excreted. There was no significant increase in excretion after carbon tetrachloride, nor was the lethal dose reduced.

Since these data indicate that acetaldehyde is destroyed in the body at least four times as rapidly as paraldehyde, the failure to find acetaldehyde in the expired air of mice to which paraldehyde had been administered is not evidence invalidating the hypothesis that paraldehyde is destroyed by depolymerization.

The final experiments of the present series demonstrated the availability of paraldehyde as a precursor of acetyl groups for the acetylation of sulfanilamide. Mice were injected intraperitoneally with 1 mgm. of sulfanilamide in 1 cc. of distilled water. Part of the mice received 5 mgm. of paraldehyde in the same solution, and part received a quantity of sodium acetate equimolar with the amount of paraldehyde. The remainder received only sulfanilamide. The urine was collected individually for a period of four hours, and the free and total sulfanilamide in each sample determined by the procedure of Bratton and Marshall (10). Data on a total of 96 mice were obtained. The results are presented

in table 2   It was found that after paraldehyde administration the sulfanilamide was excreted in the acetylated form to the extent of 45 per cent, as compared to 29 per cent for the controls   Analysis of the data by means of the "t test" of Fisher (11) shows that the probability of the difference between the mean of the paraldehyde group, and of the acetate group, and the mean of the control group being due to random sampling errors is less than 0 001

It has been shown that a number of substances are capable of acting as sources of acetyl groups   Among these are pyruvate and lactate (12, 13) acetaldehyde (13) ethanol (14) acetoin and 2, 3 butylene glycol (15) and acetate (13, 14, 16) although this last finding has not been universally confirmed (15, 17)   Although this evidence may not be taken as indication that paraldehyde is normally oxidized to acetic acid, it seems reasonable to conclude that it is normally depolymerized to acetaldehyde, which is combusted in the body through some as yet unelucidated mechanism

TABLE 2

Acetylation of sulfanilamide

All drugs given by intraperitoneal injection in 1 cc of distilled water

TREATMENT	NO OF MICE	PER CENT OF SULFANILAMIDE IN URINE AS ACETYLSULFANILAMIDE
Sulfanilamide only	34	29
Sulfanilamide plus paraldehyde	36	45
Sulfanilamide plus sodium acetate	26	45

SUMMARY

The destruction and pulmonary excretion of paraldehyde by normal and carbon tetrachloride mice have been studied, and data covering the range from the largest tolerated dose to the smallest after which any excretion could be detected have been presented   The quantities excreted vary from about fourteen per cent to very small fractions of the administered dose, being, for any particular dose, greater in the mice given carbon tetrachloride than in the normals   It was found that the rate of pulmonary excretion is a function of the dose, and that the rate of destruction by normal mice is a function of the dose at lower levels, but is constant at higher ones, while the destruction is constant and much lower in mice which have been poisoned with carbon tetrachloride

Experiments have been described on the excretion and destruction of acetaldehyde by mice   It was found that this drug is very rapidly destroyed, at least four times as rapidly as paraldehyde, and that only small quantities are excreted in the expired air   Further, it was found that the rate of destruction and excretion of acetaldehyde is unaffected by the previous administration of carbon tetrachloride

Finally it has been shown that paraldehyde is capable of increasing the acetylation of sulfanilamide by mice

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APPENDIX

In connection with the recent article by Burstein (18) on the toxicology of paraldehyde, it may be noted that in the experiments of the present study it was found that lethal doses of paraldehyde and of acetaldehyde in the mouse seem to cause death by massive pulmonary hemorrhage, and that after sublethal doses the lungs typically show large areas of pulmonary hemorrhage immediately beneath the visceral pleura. These findings are similar to those reported by Burstein for the dog. It may be further noted that the hearts of the animals killed with these drugs were in a state resembling that called "systolic standstill" as seen in the frog killed with digitalis. Whether this is a primary effect, or secondary to the extreme exsanguination seen in these animals, cannot be stated. Histological examination of several of these hearts showed no lesions nor other changes save that the nuclei seemed to be somewhat nearer each other, as might be expected.

STUDIES ON PHYSOSTIGMINE AND RELATED SUBSTANCES[1]

II THE DESTRUCTION OF PHYSOSTIGMINE IN BUFFERED SOLUTIONS AND IN SERUM

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Physostigmine and the substances pharmacologically related to it exert their biological effects by a mechanism which appears to be explainable largely if not entirely on a simple biochemical basis The history of the search for this type of an explanation for the mechanism of action of pharmacological agents starts with the discovery of Loewi (1) that physostigmine is able to prevent the hydrolysis of acetylcholine by inhibiting the enzyme cholinesterase

The multitude of papers dealing directly or indirectly with the pharmacological and cholinesterase inhibiting action of physostigmine is notably lacking in quantitative data on any one phase of the action of this important alkaloid   With the exception of the paper of Fuhner (2) in which he used the leech muscle prep aration to determine physostigmine, and the papers of Krantz and Slama (3) and Bridel (4) which deal in a qualitative way with the destruction of the drug in various solutions, we were unable to find any publications as a source of data which we needed for comparison with the data reported in this paper   It is of interest that Hesse (5) in 1867 had already observed the dependence of the formation of rubreserine (i e, the decomposition of physostigmine) upon the hydroxyl ion concentration when he found that the leech muscle color of a de composing physostigmine solution developed in a few hours in sodium bicar bonate solution, in a few minutes in sodium carbonate solution, and immediately in ammonium hydroxide solution

It was decided to make *in vitro* a series of sufficiently quantitative experiments on the action upon serum cholinesterase and on the destruction of physostigmine so that the results could be used as a basis for the control and interpretation of biological experiments

This paper reports (1) a quantitative method for the determination of physostigmine depending upon the relation of cholinesterase activity to physostigmine concentration in serum, (2) a quantitative colorimetric method for the determination of physostigmine, (3) the destruction of physostigmine in buffered solutions and in serum, and (4) the discussion of this destruction from the standpoint of its mechanism and kinetics

1 *A method for the quantitative estimation of physostigmine at low concentrations*
There has been little work done on the estimation of physostigmine at the low

mine concentration curve for the standard horse serum.   These results are shown in figure 2, which is a plot of cholinesterase activity in the final reaction mixture vs. time.   These values of cholinesterase activity were converted to physostigmine concentration in the reaction mixture from the graph of cholinesterase activity and concentration in horse serum.   The concentration in the buffer solution was obtained by multiplying the reaction mixture concentration by the proper factor, according to the degree of dilution.

B. The experiments on physostigmine destruction were extended and amplified when a quantitative method was found for the colorimetric determination of physostigmine by its conversion to the colored rubreserine.   Since this method
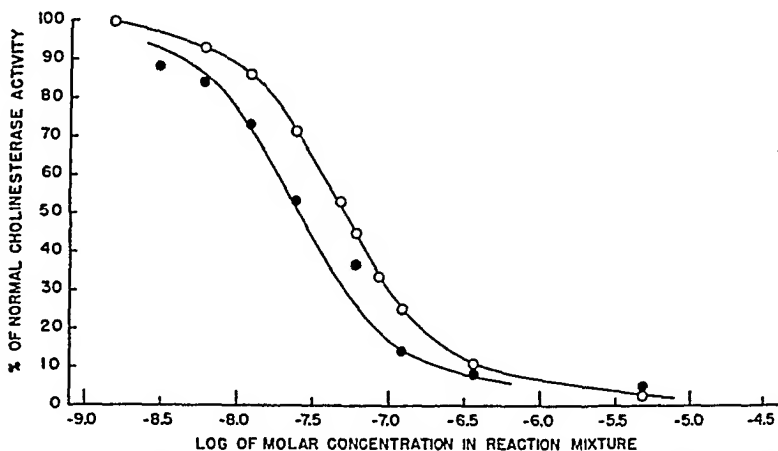
FIG. 1. CURVE OF THE PER CENT OF NORMAL CHOLINESTERASE ACTIVITY PLOTTED AGAINST LOGARITHM OF THE MOLAR CONCENTRATION OF PHYSOSTIGMINE IN THE FINAL REACTION MIXTURE

The final reaction mixture contained 22.2% serum and 2.7% acetylcholine bromide.
O = horse serum (normal activity = 213 mM/l/hr.)
● = horse serum diluted 1:2 with bicarbonate-Ringer solution (normal activity = 109 mM/l/hr.)
(Activities expressed as millimoles of acetylcholine hydrolyzed at maximal rate by one liter of serum in one hour at pH 7.4 and 38°C.)

depends upon the quantitative conversion of physostigmine to rubreserine and since eseroline is rapidly converted to rubreserine in water solution in the presence of oxygen, it was possible to determine the decomposition (hydrolysis) of physostigmine by following the reaction colorimetrically.   Thus it was possible to obtain more conveniently the rates of destruction of physostigmine at concentrations of about 10^{-3} M in solutions of various hydrogen ion concentrations for comparison with the data obtained by the enzymic method at the very low concentrations.

Colorimetric estimation of physostigmine concentration: This method determines 0.05 to 0.5 mgm. of physostigmine with an accuracy of ±2%. Larger amounts may be used if they are properly diluted so that 1 cc. contains an amount in the

stated range   The lower limit of the method is 0 01 mgm , but the accuracy is
poor in this range   Since the total volume in the determination is 5 cc , the best
range of concentration is between 0 01 and 0 10 mgm physostigmine salt per cc

The reagents needed are 10% NaOH, HCl (concentrated HCl diluted with an
equal volume of distilled water), solid NaHCO₃, and M/15 phosphate buffer,
pH 7 0   The determination is most conveniently carried out in a Klett tube
graduated at 5 and 10 cc   Known solutions gave the expected straight-line
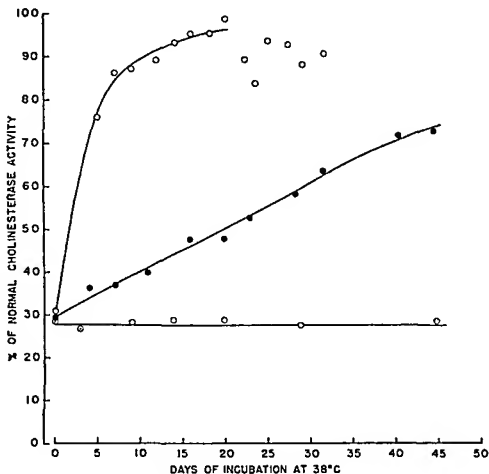
Fig 2 Incubation of Physostigmine Salicylate 1 5 × 10 ⁻⁶ M in Phosphate Buffers
at Various pH

The points are the % of normal activity of the standard horse serum in the diluted
physostigmine solution (1 15 in the final reaction mixture) at the specified time of incuba-
tion at 38°C
        O = pH 5
        ● = pH 6 8
        O = pH 8 1

relation between concentration and Klett Summerson clinical colorimetric
readings (11)

PROCEDURE   1 cc of the solution containing between 0 05 and 0 5 mgm of
physostigmine salt or the solid material diluted to contain such an amount per cc
is accurately pipetted into the reaction tube   One drop of 10% NaOH is added
and the solution shaken frequently during a period of one half hour   At the end
of this time the color development appears to have reached its maximum   One

drop of the hydrochloric acid solution is added and the mixture neutralized by adding small amounts of solid $NaHCO_3$.   The volume is then made up to 5 cc. with the phosphate buffer to prevent large changes in pH which have an effect on the color of rubreserine.   The light absorption is then determined and the concentration read from a graph of concentration of physostigmine versus colorimeter reading obtained with known concentrations of physostigmine.

In table 1 are a few examples of determinations of solutions of physostigmine (both sulfate and salicylate).   Some of the solutions which were analyzed were made up by others so that the concentrations were unknown at the time of analysis.

TABLE 1

Physostigmine determinations using the colorimetric method

COLORIMETER READING	PHYSOSTIGMINE SULFATE	
	Graphically determined concentration	Actual concentration
	mgm./cc.	*mgm./cc.*
330*	5.0	5.0
325	0.100	0.100
330	0.101	0.100
195	0.059	0.060
197	0.060	0.060
131	0.039	0.040
137	0.041	0.040
76	0.022	0.020
75	0.021	0.020
45	0.012	0.010
41	0.011	0.010
113	0.042†	0.040†
34	0.010†	0.010†

* For this determination the solution was diluted 1:50.

† Physostigmine salicylate.

C. The data obtained by these two entirely unrelated methods over a range of concentration differences of 1000 and at various hydrogen ion concentrations from pH 5 to 8.1 can be used to show that the mechanism of the destruction of physostigmine in buffered solutions is given by the following equation:

$$Physostigmine + (OH^-) + H_2O \longrightarrow (OH^-) + Split\ Products$$
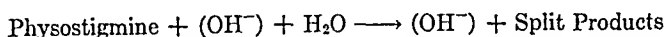
Since these reactions were run in dilute solutions, water was in excess and thus its concentration did not change significantly.   Also, in buffered solutions the hydroxyl ion concentration during each experiment was kept constant.   Therefore, according to this equation, the rate of reaction at any one temperature must depend upon the concentration of physostigmine and the hydroxyl ion concentration.   The reaction thus appears as a second order reaction in which one of the
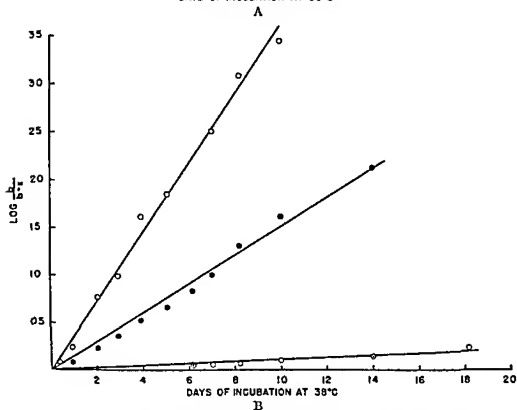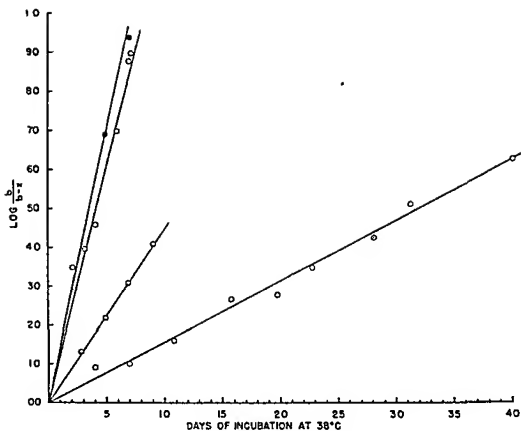
FIG 3 GRAPHS OF DESTRUCTION OF PHYSOSTIGMINE IN BUFFERS OF VARIOUS pH
See text for details

A Enzymic method of analysis
O = pH 6 8
O = pH 7 2
O = pH 7 8
● = pH 8 1.

B Colorimetric method of analysis
O = pH 6 0
● = pH 7 0
O = pH 7 3

reactants has been conveniently kept constant. Mathematically the destruction is given by the following equation:

$$\frac{dx}{dt} = k \text{ (Physostigmine)(OH}^-)$$ Equation 1

According to Taylor (12) this type of bimolecular reaction in which one of the reactants is constant can be integrated to the following equation:

$$k = \frac{2.3}{at} \log \frac{b}{b - x}$$ Equation 2

in which a is the concentration of hydroxyl ions, b is the concentration of physostigmine at zero time, x is the amount of physostigmine destroyed in t hours.

TABLE 2

Destruction of physostigmine in buffered solutions

pH	PHYSOSTIGMINE CONCENTRATION	METHOD OF ANALYSIS	SLOPE OF LINE LOG $\frac{b}{b-x}$ VS t	VALUE OF $\frac{2.3}{a}$	DESTRUCTION CONSTANT, $K \times 10^4$
					liter/mol /hr.
6.0	3.09×10^{-3}	Colorimetric	4.5×10^{-5}	2.3×10^8	1.04
6.8	1.5×10^{-6}	Enzymic	6.6×10^{-4}	3.65×10^7	2.41
7.0	3.09×10^{-3}	Colorimetric	6.3×10^{-4}	2.3×10^7	1.45
7.2	1.5×10^{-6}	Enzymic	1.9×10^{-3}	1.4×10^7	2.66
7.3	3.09×10^{-3}	Colorimetric	1.5×10^{-3}	1.15×10^7	1.73
7.8	2.02×10^{-5}	Enzymic	5.1×10^{-3}	3.65×10^6	1.86
8.1	1.5×10^{-6}	Enzymic	5.9×10^{-3}	1.77×10^5	1.04
Average..................					1.73

When the term $\log \frac{b}{b-x}$ is plotted against t, a straight-line relation should be obtained, with its origin at zero, if the reaction does follow the mechanism put forth by this equation.   The graphs of figure 3 A and B give the data on the reactions at various hydrogen ion concentrations determined by the enzymic and colorimetric methods.   The destruction at pH 5 and 38°C. was found to be immeasurably slow.   It is obvious that straight lines are obtained and the reactions thus appear to follow a second order mechanism.   When k is calculated for each of the reactions by multiplying the slope of the line by the factor $\frac{2.3}{a}$, it appears to be relatively constant in view of the extreme differences in concentration and methods of analysis used.   The value of k for separate reactions together with pertinent data on the reactions are listed in table 2.

3. *The destruction of physostigmine salicylate in horse serum at 38°C.*   Various amounts of physostigmine salicylate solution were added to sterile horse serum so that the added volume amounted to less than 1% of the serum, and the mixture was incubated at 38°C.   Controls were run by adding the same volume of dis-

tilled water to serum   Serum at 38°C retained its original cholinesterase activity for at least 72 hours   Bacterial cultures were made to substantiate the sterility of the mixtures   At intervals, after mixing the drug and serum, 1 cc samples were taken and the cholinesterase activity of each sample was determined   Typical results of three of these experiments are shown in figure 4, in which percent of normal cholinesterase activity in the final reaction mixture is plotted against time   The activity values of figure 4 were converted to physo-
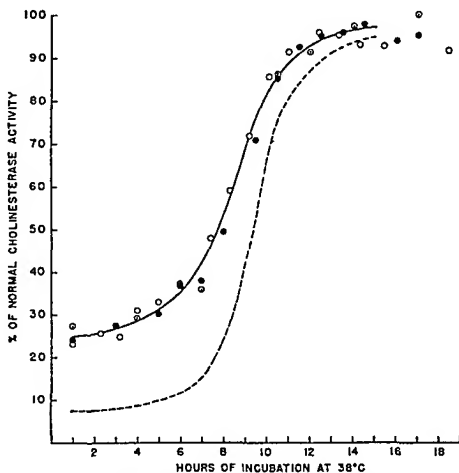
FIG 4 DESTRUCTION OF PHYSOSTIGMINE IN HORSE SERUM [NORMAL CHOLINESTERASE ACTIVITY = 247 mM/l/hr   (See legend of figure 1)]

stigmine concentration in the reaction mixture by the use of the horse serum curve of figure 1   The true physostigmine concentration of serum was obtained by multiplying the reaction mixture concentration by a factor of 4 5 which corrects for the degree of dilution of the serum

Cholinesterase activity has been shown to be related to the total concentration of physostigmine and also the concentration of the enzyme [see fig 1 and Straus and Goldstein (13)]   The relation between the activity and concentration can be

used to determine the dissociation constant of the enzyme-inhibitor complex, and also to determine the concentration of the enzyme. This is accomplished by determining the factor i, which is the fractional inhibition, at various concentrations of I, which is the total concentration of the inhibitor in the reaction mixture. By the use of the equation derived by Easson and Stedman (14) relating the enzyme concentration, the total inhibitor, and the fractional inhibition, it is
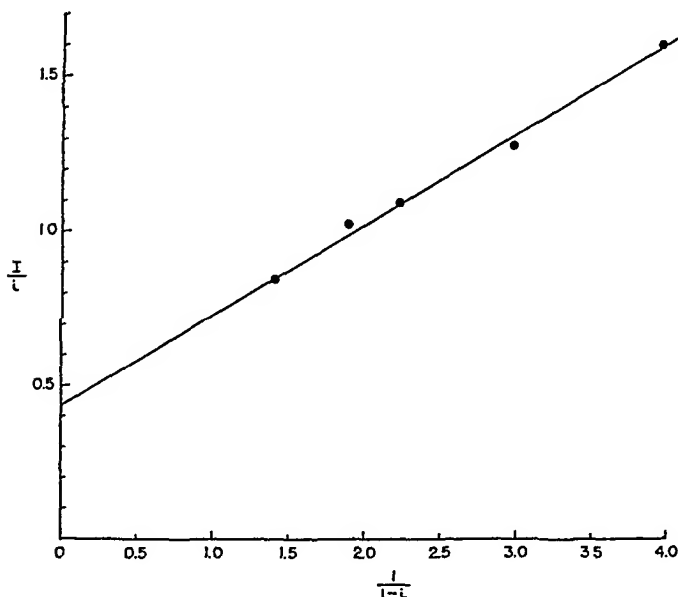
FIG. 5. GRAPH OF THE RELATION OF TWO TERMS IN THE EQUATION $\frac{I}{i} = K\frac{1}{1-i} + E$.

ORDINATE $= \frac{I}{i} \times 10^{-7}$; ABSCISSA $= \frac{1}{1-i}$.

The slope and intercept are the values of K ($= 3.0 \times 10^{-8}$), and E ($= 0.44 \times 10^{-7}$), respectively. The value of E is for the concentration in the final reaction mixture.

possible to obtain graphically the enzyme concentration and the dissociation constant. Their equation is

$$\frac{I}{i} = K\frac{1}{1-i} + E$$

From this equation it is expected that a graph in which the term $\frac{1}{1-i}$ is plotted against $\frac{I}{i}$ should give a straight line. The slope and intercept of the line are the values of K and E, respectively. This is shown in figure 5, in which the values of i and I are those for the final reaction mixture, and from this graph we obtained the values of 0.44×10^{-7} for E, the enzyme concentration (active centers), and

3.0×10^{-8} for K, the dissociation constant   Since the reaction mixture is a 1 4 5 dilution of horse serum, the enzyme concentration in the serum is 2.0×10^{-7} M

In an attempt to simplify the description of the mechanism for the inactivation of physostigmine in serum and to find a common denominator for the decomposition of physostigmine in buffered solution and in serum we set up the equation for the reaction of physostigmine and cholinesterase as follows

$$E + I \rightarrow E + SP$$
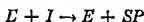
in which E is the cholinesterase concentration, I is the physostigmine concentration, and SP is the concentration of split products formed in the decomposition of physostigmine   Although this is a hydrolytic reaction, water is not considered in the equation, since it is in great excess and can thus be considered constant   Under the conditions of the experiment there is no significant change in the total cholinesterase concentration   Thus the reaction simplifies to exactly the same mechanism as the hydrolysis in buffered solution with the enzyme concentration replacing the hydroxyl ion concentration   In other words, it is a second-order reaction in which one of the reactants remains constant throughout the course of the reaction   This is merely a restatement of the ordinary type of enzymic reaction in which the substrate is not in excess

In the treatment of this data on the enzymic decomposition of physostigmine we have used the same method as was used in the study of the hydrolysis of this drug in buffered solution   In order to determine the rate of reaction it was necessary to plot the term $\log \dfrac{b}{b-x}$ against time   In figure 6 we have plotted these data on the destruction of physostigmine on the basis of the reaction following the mechanism of a simple enzyme substrate reaction in which the substrate is not in excess   The reaction does appear to follow this mechanism after the sixth hour   This allows a calculation of the rate constant from the slope of the straight line drawn through experimental points following this time   The slope is 0 238   The enzyme concentration is 2.0×10^{-7} molar and thus the value of $\dfrac{2.3}{a}$ is 1.15×10^7 Therefore, the value of K, the destruction constant, is 2.74×10^6

The slow rate at the beginning of the reaction is probably accounted for by the high concentration of physostigmine during this part of the reaction   Although the combination of physostigmine and cholinesterase is slow in comparison with the rate of combination of other enzymes with their substrates (such as acetylcholine and cholinesterase), the equilibrium is reached in about one hour   This statement is based on some preliminary data obtained by determining the activity of either horse or dog serum at several periods during the first few hours after mixing the serum with a solution of physostigmine   The most important factor in the early slow rate of inactivation appears to be some sort of inhibition of the reaction by high concentrations of physostigmine   This was brought out when the data was plotted as the velocity of reaction at various physostigmine

concentrations.   When treated in this way, the rate of destruction increases with increasing physostigmine concentration to an optimum velocity and then decreases at higher concentrations of the substrate.   Experiments with very high physostigmine concentrations showed a very slow recovery in cholinesterase activity until the activity approached 20% of the normal value.   The recovery from this level followed the time course for a pseudomonomolecular reaction. The same inhibition of destruction at high concentrations of prostigmine was noted when the data of Easson and Stedman (14) were plotted on the same basis. Similar results on esterases were reported by Bamann and Schmeller (15), and
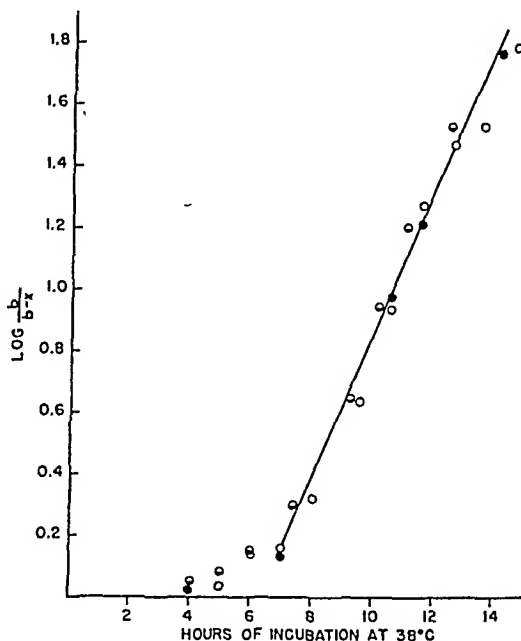
FIG. 6. GRAPH OF THE DATA OF THE DESTRUCTION OF PHYSOSTIGMINE IN HORSE SERUM OF FIGURE 4 PLOTTED AS A SECOND-ORDER REACTION

later confirmed by Murray (16), who gave a mathematical treatment of his findings.   Lineweaver and Burk (17) have given graphical methods for treating enzyme reactions which are inhibited by excess substrate.   However, our results at high inhibitor concentration are insufficient for the treatment they proposed.

From our data we are able to state that the destruction of physostigmine in serum may be calculated from the rate constant given above if the cholinesterase concentration, the dissociation constant, and the inhibition in the system are determined.   This will describe the destruction when the percentage activity of the cholinesterase is not less than 15 to 20% of the uninhibited value.   At lower activities the reaction is too complicated for a simple description and must await

further experimental work undertaken for the express purpose of analyzing the reaction at these very low cholinesterase activities

DISCUSSION   The only quantitative results on the destruction of similar enzyme inhibitors with which we have been able to compare our data are those of Easson and Stedman (14), who studied miotine in a preliminary way and made a detailed study of prostigmine inactivation by solutions of purified cholinesterase in order to determine the absolute activity of cholinesterase

The rate of decomposition of prostigmine by cholinesterase as studied by Easson and Stedman was determined by the use of the experimentally determined values of z and the rather complicated equation which follows

$$\log_e \frac{z_2}{z_1} = \frac{K}{[E]} \left\{ \log_e \frac{z_1(1 - z_2)}{z_2(1 - z_1)} + \frac{z_1 - z_2}{(1 - z_1)(1 - z_2)} \right\} + \frac{-k}{[E]} (t_2 - t_1)$$

From this equation they plotted the value $\left\{ \log_e \frac{z_1(1 - z_2)}{z_2(1 - z_1)} + \frac{z_1 - z_2}{(1 - z_1)(1 - z_2)} \right\}$

against $\log_e \frac{z_2}{z_1}$ from experiments in which $(t_2 - t_1)$ was kept constant, and found that the points lay on a straight line with the exception of the lowest and the two highest values obtained   From this straight line they were able to determine the

ratio $\frac{K}{E}$, which was equal to the slope, and the term $\frac{-k}{[E]}$ which was the intercept   From these ratios and the previously determined values of K and E, the value of the destruction constant was obtained

When we attempted to use the same method in handling our data on the decomposition of physostigmine in serum, it was found that only a few of the points lay on the part of the graph which would be expected to give a straight line relation   Most of the points gave an impression of something approaching a triangle   The graph of Easson and Stedman is somewhat similar   The high and low values gave points far off the straight line which was drawn   The results of Easson and Stedman's experiments appear to be more suitable to their graphical treatment than do our own   This is, we believe, due to their determination of only a few points, the majority of which fell into the fortuitous range in which the points seem to form about a straight line

SUMMARY

1 A method is reported for the quantitative estimation of physostigmine at concentrations between 10^{-6} and 10^{-8} molar   This method depends upon the quantitative relation of the inhibition of serum cholinesterase activity to the physostigmine concentration in the above range of inhibitor concentration

2 A colorimetric method for the estimation of physostigmine is described

3 The decomposition of physostigmine in buffered solutions at pH 5–9 has been studied   The rate of destruction is a reflection of the physostigmine and hydroxyl ion concentrations

4 The rate of serum inactivation of physostigmine has been shown to be related to the concentration of both the drug and the cholinesterase activity when

the serum activity is greater than 15–20% of the normal uninhibited activity. When the physostigmine concentration is sufficient to depress the cholinesterase activity more completely, the rate of physostigmine destruction is slow. This is interpreted as an inhibition due to excess substrate (physostigmine).

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STUDIES ON PHYSOSTIGMINE AND RELATED SUBSTANCES[1]

III BREAKDOWN PRODUCTS OF PHYSOSTIGMINE, THEIR INHIBITORY EFFECT ON CHOLINESTERASE AND THEIR PHARMACOLOGICAL ACTION

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In a series of experiments from this laboratory the destruction of physostigmine *in vitro* was studied in various media (1)   It was possible to determine changes in physostigmine concentration by estimating the percentage change of cholinesterase activity of a standard sample of serum in the presence of this alkaloid, the concentration of which under standard conditions, shows a quantitative relation to cholinesterase activity (see figure 2 physostigmine curve)   It was observed in these experiments that inhibitory activity reappeared in solutions of physostigmine which were tested for their action on cholinesterase activity after the original inhibitory activity of the solution had been lost   For instance, when the rate of breakdown of physostigmine in phosphate buffer at physiological pH and 38°C was determined, the cholinesterase inhibition by physostigmine was completely lost in 30 days, but after 60 days there was evidence of a return of some inhibitory power to the solution   Similarly when the cholinesterase activity of a solution of horse serum in bicarbonate Ringer solution containing physostigmine was estimated at intervals for a period of several hours, it returned to normal in 15 to 20 hours   Later, however, the cholinesterase activity decreased to 90% of the normal (fig 1)

From these observations it was postulated that some of the breakdown products of physostigmine were inhibitors of cholinesterase   The clarification of this point seemed important in connection with the general problem of the metabolism of physostigmine since the body rids itself of this substance mainly by destroying the substance (2)

Some of the primary reactions in the mechanism of the decomposition of physostigmine are well established   The first reaction is a hydrolysis of the methylcarbamyl group and the formation of eseroline   There follows an oxidation during which eseroline is converted into rubreserine   Rubreserine was shown to be an ortho quinone related in structure to adrenochrome, and, as it was possible to convert it to eserine blue, it must be considered an intermediate in the formation of the blue substance (3)   Further destructive reactions result in the brown mixture called eserine brown

The only papers that have come to our attention on the biological activity of

the two important breakdown products of physostigmine, rubreserine and eserine blue, are those of Eber (4) and Heubner (5), both of whom found no significant activity in biological tests of these substances.

The compounds used in this study were prepared from physostigmine sulfate[2] by Ellis (3). Their chemical and physical properties are as follows:

Eseroline, $C_{13}H_{18}ON_2$, colorless crystals, m.p. 128°.
Rubreserine monohydrate, $C_{13}H_{16}O_2N_2 \cdot H_2O$, red crystals, m.p. 132-133°.
Eserine blue dihydrochloride, $C_{17}H_{23}O_2N_3 \cdot 2HCl$, blue amorphous substance, decomposes above 300°.
Eserine brown, a brown amorphous mixture, decomposes above 300°.
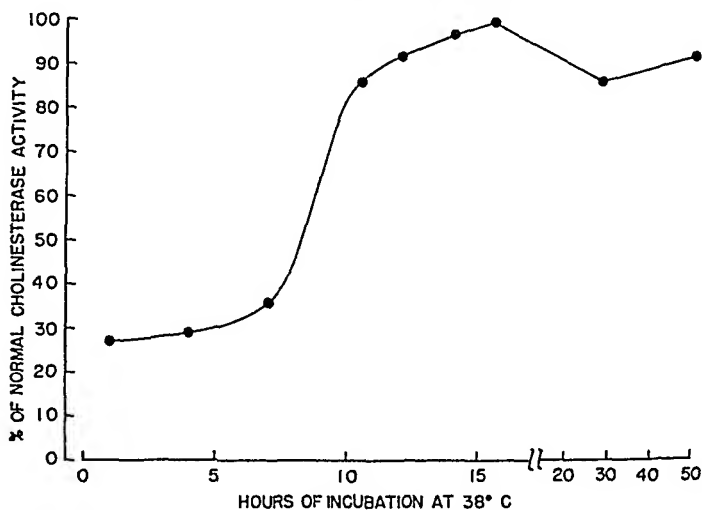
Fig. 1. Recovery of Serum Cholinesterase Activity during Incubation at 38°C. of 20 cc. of Horse Serum Mixed with 0.4 cc. of 1:100,000 Physostigmine Salicylate

The experimental points are the per cent of the normal activity in the final reaction mixture, which was a dilution of 1:4.5 of the serum-physostigmine solution.

1. *The effect of the breakdown products of physostigmine upon the activity of cholinesterase.* Rubreserine, eserine blue, eseroline, and eserine brown were tested as to their influence upon the activity of cholinesterase of horse serum *in vitro* by the method of Friend and Krayer (6). The substance under investigation was dissolved in bicarbonate Ringer solution and the concentration calculated on the basis of the amount in the final reaction mixture.

Rubreserine and eserine blue proved to be inhibitors of cholinesterase. In order to rule out the possibility that the inhibitory action of the compounds was due to contamination with the much more potent physostigmine, the activity of the preparations were determined at various concentrations. The results are

[2] The physostigmine salicylate used in these experiments was generously supplied to us by Merck & Co., Inc., Rahway, N. J.

reported in figure 2   A comparison of the slopes of the curves obtained for rubreserine and eserine blue with that for physostigmine demonstrates rather conclusively that the enzymic inhibition caused by rubreserine and eserine blue was not due to contamination with physostigmine   From the data of figure 2 obtained in a final reaction mixture containing 22 2% horse serum, inhibition to 50% of the normal activity was produced by the following concentrations in 1 cc of the reaction mixture   physostigmine salicylate, 0 02 microgram (4×10^{-8} molar)   rubreserine, 2 3 micrograms ($9 9 \times 10^{-8}$ molar), eserine blue, 1 7 micrograms ($5 7 \times 10^{-8}$ molar)   (Physostigmine sulfate and physostigmine salicylate showed the same activity in equimolar concentrations)

A further proof of the true activity of rubreserine is the conversion of the inactive eseroline to the active rubreserine   A solution of eseroline in bicarbonate
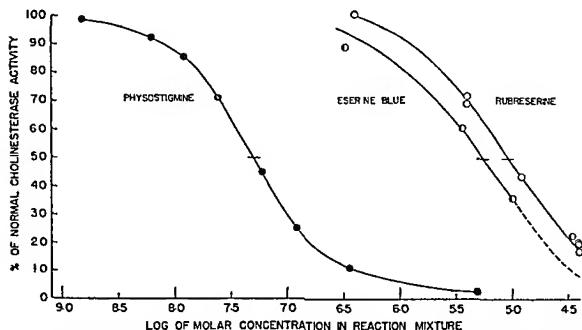
FIG 2 INHIBITION OF CHOLINESTERASE ACTIVITY IN 22 2% HORSE SERUM IN VITRO
The RELATIVE POTENCY OF PHYSOSTIGMINE ESERINE BLUE AND RUBRESERINE

Ringer solution (in a concentration such that the final reaction mixture would contain 10 micrograms per cc) was colorless and did not inhibit cholinesterase   However, after standing for one day, the solution which had turned pink, de pressed the cholinesterase activity of horse serum to 90% of the normal   When kept for one month in the refrigerator below 4°C , this solution retained its pink color as well as its activity

Of several preparations of eserine brown only one showed inhibitory activity   When tested at several different concentrations, it became evident that the activity was due to contamination with eserine blue   This was later shown chemically by the typical green fluorescence which appeared in the chloroform layer when the preparation was extracted with that solvent   Furthermore, acidification of a solution of this eserine brown in bicarbonate Ringer brought out the red fluorescence characteristic of eserine blue in acid solution   Eserine

brown, therefore, must be considered devoid of inhibitory action upon cholinesterase. The highest concentration of inactive eserine brown used was 10 micrograms per cc. of reaction mixture, or four times the concentration of rubreserine leading to 50% inhibition.

2. *Biological activity of rubreserine and eserine blue.* In conformity with their effect upon cholinesterase activity *in vitro,* rubreserine and eserine blue caused pharmacological effects similar to physostigmine upon surviving biological preparations as well as upon the intact animal.

The leech muscle strip, prepared in a manner similar to Führner's technique (7), was sensitized to acetylcholine by concentrations of rubreserine (4 experiments) and eserine blue (3 experiments) which in themselves did not influence the

FIG. 3. 2/13/42. LEECH MUSCLE STRIP SUSPENDED IN RINGER SOLUTION.
At *1,* acetylcholine chloride 1:500,000; at *2,* acetylcholine chloride 1:1 million; at *3,* acetylcholine chloride 1:100 million. Between *2* and *3,* rubreserine 1:500,000 for 53 minutes. Time intervals in minutes.

activity.   Figure 3 illustrates the action of rubreserine.   The effect of acetylcholine was increased more than a hundredfold.   It is noteworthy that the concentration of 1:500,000 used was the same as that commonly used of physostigmine salicylate or sulfate.   Similarly, the rectus abdominis muscle of the frog was sensitized to acetylcholine by rubreserine (27 experiments) and eserine blue (19 experiments).

The smooth muscle preparation of the small intestine of the rabbit in the arrangement of Magnus (8) was stimulated by both substances in low concentrations such that tone and amplitude of contraction increased.   As the action of rubreserine and eserine blue (between 1:3 million and 1:2 million), as well as that of physostigmine salicylate (1:6 million), proved reversible, this method made possible a comparison of the alkaloids.   Physostigmine salicylate was approx-

imately 2.5 times as potent as rubreserine (7 experiments) and about 3 times as potent as eserine blue (8 experiments). With high enough concentrations the typical spastic state of the gut appeared; figure 4 shows the effect of eserine blue 1:600,000, as well as the characteristic prompt abolition of the action by atropine sulfate.

When 1–2 mgm. of either rubreserine (3 experiments) or eserine blue (2 experiments) was added to the blood in the heart-lung preparation of the dog [prepared according to Knowlton and Starling (9)] one-half to one hour after severing the vagi, the heart rate gradually decreased. As the total blood volume of these heart-lung preparations was about 500 cc., the concentration employed was in the range of 1:500,000 to 1:250,000. The marked drop in heart rate could be counteracted readily by the administration of 0.5 mgm. of atropine sulfate.

FIG 4. 2/16/42   ISOLATED RABBIT INTESTINE (ILEUM)
1, 0.05 mgm eserine blue, 2, 0.05 mgm atropine sulfate, 3, 0.5 mgm eserine blue   Capacity of the bath, 30 cc   Time in 1-minute intervals

In the intact cat two drops of a solution of 1:1,000 of either rubreserine or eserine blue (two experiments each) instilled into the conjunctival sac caused a miotic effect which began after 15 minutes and led to a complete contraction of the pupil within 60 minutes   The pupillary effect could be abolished in the characteristic way by local administration of 1 to 2 drops of atropine sulfate 1:1,000. Rubreserine 1:1,000 was also given into the eye of two rabbits and caused a constriction of the pupil lasting for several hours

Neuro-muscular effects. Seven experiments, 1 with rubreserine, 2 with eserine blue, and 4 with physostigmine sulfate, were done on 5 cats under dial[3] anesthesia

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[3] Dial contains 0.1 gram of dial and 0.4 gram of urethane per cc   This mixture was generously supplied to us by Ciba Pharmaceutical Products Inc

(0.7 cc. per kgm. body weight intraperitoneally) with uniform results.   The sciatic nerve was cut above the hip, and shielded electrodes were then applied to it at the level of the hip.   Single supramaximal condenser discharges of brief duration were delivered to the nerve for long periods at a rate of one per 4.2 seconds.   The twitches of the calf muscles were recorded on a drum from the Achilles tendon, which was dissected free and tied to a myograph.   After a few minutes of stimulation the height of the twitch became steady and intravenous injections of 0.1 mgm. per kgm. of physostigmine sulfate produced an easily recog-
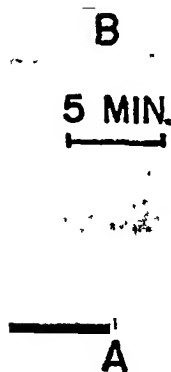
FIG. 5. EFFECT OF RUBRESERINE ON MAXIMAL TWITCH HEIGHT OF CIRCULATED CAT CALF MUSCLE COMPARED WITH THAT OF PHYSOSTIGMINE
See text for details of preparation.   At A, 1 mgm. of rubreserine per kgm. intravenously At B, 4 hours later, 0.3 mgm. of physostigmine sulfate per kgm.

nized increase in twitch height (10).   The injection of eserine blue or rubreserine in doses of 1 to 1.5 mgm. per kgm. elicited somewhat less increase in twitch height than 0.3 mgm. per kgm. of physostigmine sulfate (fig. 5).

As a marked increase in pendular movements and in propulsive activity of the gut was observed in a cat as well as in a rabbit with abdominal window under anesthesia after repeated injection of 1 mgm. of rubreserine, experiments were made to test the effect of rubreserine and of eserine blue on rabbits and dogs without anesthesia.   In table 1 is recorded the time course of two experiments with rubreserine given intravenously.

In two other rabbits increased intestinal activity resulted in the passage first of formed and later of fluid feces after the administration of a total amount of 1 5 mgm of rubreserine per kgm body weight in 3 equal doses within 30 minutes Eserine blue given in a dosage of 0 5 mgm per kgm to a dog had no noticeable effect upon gastrointestinal activity and was also inactive in two rabbits, one given 0 5 mgm per kgm in a single dose and the other a total amount of 1 9 mgm per kgm in 3 doses within 45 minutes

TABLE 1

Effect of rubreserine on normal mammals

TIME	REMARKS
Rabbit 2 11 kgm	1 mgm rubreserine injected into ear vein at zero time (time in minutes after injection)
3	Some gnawing movements
7	Passes formed feces gnawing movements continue
9	Passes thin feces
11	Gnawing movements continue, ears drooping
12	Passes liquid feces, very marked gnawing movements
33	Animal has been sitting throughout Swallowing movements from time to time little motor activity
56	No noticeable change
Next day	Animal shows no after effects
Dog 25 7 kgm	10 mgm rubreserine injected into vein of hind leg at zero time (time in minutes after injection)
2	Scratching violently
6	Salivation continues to scratch wildly
7	Marked salivation, passes large amount of formed feces Scratching less
9	Passes small amount of feces after repeated unsuccessful attempts
10	Marked salivation continues Passes liquid feces Scratching has subsided
13	Passes liquid feces
16	Lying down Salivation still increased
30	Lying down Marked salivation
47	Animal more active, some salivation
100	Animal appears normal

3 *Comparison of biological effect of rubreserine and eserine blue with earlier work.* The results of our experiments on the biological activity of some of the breakdown products of physostigmine, especially of rubreserine, are at variance with the reports in the literature Heubner (5) found the subcutaneous injection of 20 mgm and 45 minutes later of 60 mgm of rubreserine to an 11 kgm dog without effect Likewise in a rabbit of 2 kgm kept in a saline bath at 37°C with the abdomen opened for observation of intestinal activity, 40 mgm of rubreserine given intravenously was without effect Similarly 12 mgm of eserine

blue given intravenously to a normal rabbit of 2.15 kgm. within 10 minutes had no noticeable action; only in the frog eserine blue had a paralyzing action upon the heart and upon the central nervous system.   This discrepancy required an *examination of the detailed chemical methods* used by Heubner for the preparation of the material used in his biological experiments, but unfortunately his description is not given in sufficient detail.   It would appear that to prepare rubreserine Heubner heated for up to three days at 80 to 90°C. in air or oxygen a solution of physostigmine sulfate which had been converted to free physostigmine by the addition of barium hydroxide.   The resulting red solution was then made alkaline and extracted with chloroform.   Our attempts to duplicate Heubner's procedure resulted in a blood red solution at the end of the three-day period, but, contrary to Heubner, who recovered only rubreserine, the material extracted by us proved to be mainly eserine blue, a substance which Heubner knew to show a red fluorescence.   The dried residue of the three-day sample was found to be practically inactive when injected intravenously into dogs in a dose as high as 2.0 mgm. per kgm.

The conversion of physostigmine to its breakdown products under Heubner's conditions was then followed colorimetrically, using the method developed by Ellis (1), and conversion of the physostigmine appeared to be complete in about ten hours.   Samples taken at 5 and 10 hours, extracted with chloroform, and dried *in vacuo* contained much rubreserine, and the 10-hour sample contained more eserine blue than the 5-hour one.

A comparison of the 5-hour, 10-hour, and 3-day samples in dogs gave the following result: The material from the 5-hour sample was injected intravenously into two dogs, to one in a dose of 0.85 mgm. per kgm. body weight, followed in 50 minutes by a second dose of 1.25 mgm. per kgm., to the second in a single dose of 1.7 mgm. per kgm.   The animals became somewhat restless, began to scratch rather wildly, as was noted in table 1, and continually, for some time, rubbed their bodies against the wall and floor of the cage.   No other effect was observed.   The 10-hour sample when given in a dose of 0.8 mgm. per kgm. to one dog caused salivation, and very shortly the mouth of the animal was full of froth; difficulty in breathing led to a marked cyanosis.   The animal became ataxic, unable to walk, and began to tremble as though it had slight clonic convulsions.   Feces were passed repeatedly.   Within one hour recovery was nearly complete.   A dose of 1.15 mgm. per kgm. in a second dog rapidly caused salivation and frothing.   Clonic convulsions appeared, together with marked difficulty in breathing.   Deep cyanosis developed and the animal died 11 minutes after the injection from asphyxia in a convulsive attack.   1.9 mgm. per kgm. in a third dog caused the same symptoms as in the second animal.   Atropine sulfate given 15 minutes after the injection relieved the difficulty in breathing and the cyanosis disappeared.   The convulsive attacks continued but could be suppressed by ether anesthesia.   Anesthesia was kept up for about 30 minutes, and the animal subsequently recovered.

The 3-day sample given to three dogs in a dose of 0.8, 0.85, and 2.0 mgm. per kgm. was similar in its effect to the 5-hour sample in regard to the initial motor

excitement and scratching, but subsequently two of the animals appeared more depressed. In only one dog (0 3 mgm. per kgm.) defecation was observed several times. The urine of the animal receiving 0 35 mgm./kgm. was collected for 20 hours, and 2% of the total amount of material administered was recovered as eserine blue from the urine.

The toxicity of the 5 hour, 10 hour, and 3 day sample was tested in mice, giving three graded doses of each substance to groups of 6 animals each by injection into the tail vein. The approximate L D 50 in mgm. per kgm. of body weight was as follows: 5 hour sample, 1 5; 10 hour sample, 1 2; 3 day sample, 12 0. In comparison, physostigmine sulfate had an L D 50 of 0 25, rubreserine of 2 0, and eserine blue of 2 0 mgm. per kgm.

DISCUSSION   A few reports have appeared in the literature on the biological activity of products obtained from physostigmine. Thus, rubreserine and eserine blue were found by Eber (4) to be inactive. Salway (11) reported that physovenine, an unusual monoester of carbonic acid, which was claimed to be formed when only the methylamine group of physostigmine was hydrolyzed, was a powerful miotic. Since this compound readily formed rubreserine, it appears to us that the activity may have been due to that compound. Schweitzer, Stedman, and Wright (12) found that eseroline hydrochloride showed a slight inhibition of cholinesterase *in vitro* in a concentration of 5×10^{-4}, whereas physostigmine sulfate was active at 10^{-8} molar. They also reported that eseroline showed a feeble stimulation of spinal reflexes *in vivo* with an action about $\frac{1}{50}$ of that of physostigmine. We found it practically impossible to test eseroline in a concentration greater than 1 100,000 at physiological pH. Solutions with higher concentrations rapidly turned pink because of the formation of rubreserine in an amount sufficient to cause enzymic action. The reports on the activity of eseroline at physiological pH with no special precautions taken to prevent oxidation are therefore questionable.

The strong inhibitory effects on cholinesterase shown by rubreserine and eserine blue are remarkable when it is considered that these compounds do not contain the carbamyl ester linkage which Stedman (13) assumed to be essential for an efficient inhibition of cholinesterase. It has been shown, however, that substances which do not have the carbamyl group, such as strychnine (14), atropine (15), and morphine (16), are inhibitors of cholinesterase, although their activity is very low compared with physostigmine or prostigmine. More recently Rentz (17) reported that methylene blue *in vitro* as well as *in vivo* inhibited cholinesterase activity almost as powerfully as did physostigmine, while trypaflavine he found to be a less powerful inhibitor. Trypaflavine is in about the same range of potency as rubreserine and eserine blue. The results of Rentz are in accord with those of Massart and Dufait (18), who state that basic dyes inhibit cholinesterase, whereas acid dyes do not.

Our experiments on isolated organs and on normal animals indicate that rubreserine and eserine blue are pharmacologically active substances and that their actions can at least partly be explained on the basis of their cholinesterase inhibiting properties. We cannot satisfactorily explain why Heubner was

unable to observe such activity. In the first place, it is not possible from his statements to be certain about the purity of his rubreserine and eserine blue. Furthermore, his negative evidence is based essentially upon the results of two experiments, one with a normal dog, the other with a rabbit. If Heubner used a preparation similar to our 3-day sample of breakdown products, rather than pure rubreserine, as he assumed, then the subcutaneous injection of 2.7 mgm. per kgm. followed 45 minutes later by 5.6 mgm. per kgm. of body weight in his dog experiment can well have been just below the effective dose, as we have shown 2 mgm. per kgm. of the 3-day sample to be without effect when injected intravenously. Heubner's negative evidence, then, rests with an experiment in a single rabbit in which he injected intravenously 20 mgm. of his rubreserine per kgm. without effect. This does not agree with the L.D. 50 in mice of 12 mgm. per kgm. even for our 3-day sample and still less with the higher toxicity of the 5- and 10-hour samples and the effect of our rubreserine.

SUMMARY

1. Rubreserine and eserine blue are strong inhibitors of cholinesterase. On serum cholinesterase *in vitro* their potency on a molar basis is 1/100th that of physostigmine. Eseroline and eserine brown are devoid of this action.

2. Rubreserine and eserine blue sensitize the leech muscle strip and the rectus abdominis muscle of the frog to acetylcholine. They cause an increase in amplitude of contraction and tonus of the isolated small intestine of the rabbit, a decrease in heart rate of the isolated, denervated mammalian heart, and a constriction of the pupil of the eye of the cat and rabbit. The action upon the isolated gut, heart rate, and pupil of the eye can be abolished promptly and completely by atropine.

3. The maximal twitch height of the calf muscle of the cat in response to single, brief supramaximal condenser discharges was increased by rubreserine and eserine blue in a similar way as with physostigmine.

4. Experiments are reported on the pharmacological action of rubreserine and eserine blue upon normal rabbits and dogs after intravenous injection and on the toxicity of rubreserine, eserine blue, and physostigmine in mice after intravenous injection.

5. Reasons are discussed for the failure of previous investigators to show the pharmacological properties of rubreserine and eserine blue.

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BLOOD LEVELS OF SULFADIAZINE, SULFAMERAZINE AND SULFAMETHAZINE IN RELATION TO BINDING IN THE PLASMA

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Comparative studies of the absorption, excretion and blood levels after single doses of sulfadiazine, sulfamerazine and sulfamethazine in man have been reported recently (1, 2).   Calculations from these data show that the blood and plasma levels of all these drugs are higher than would obtain if the amounts of drugs present in the body at a given time were equally distributed throughout the body water.   For sulfadiazine, the concentrations found in the plasma when given amounts of drug were present in the body (1, 2) accord with the fact that about 55 per cent of the plasma sulfadiazine is not dialyzable, being bound to plasma protein (3, 4, 5).   For a given amount of drug in the body, the plasma levels of sulfamerazine and sulfamethazine are considerably higher than for sulfadiazine (1, 2, 6, 7), suggesting that these drugs are bound in the plasma to a greater extent than is sulfadiazine.

To test this hypothesis, comparative *in vitro* dialysis studies of the extent of binding in the plasma of these three drugs and of their acetyl derivatives[1] were ·made.   The results of these *in vitro* studies have been compared with the "*in vivo*" binding of the drugs in the plasma, calculated as described below from available data for absorption, excretion and blood levels after single doses of the drugs in man (1, 2, 6, 7).   The distribution of the compounds between plasma and red cells were also studied.

The relative extent of binding of these sulfonamides would appear to be important in respect to the relative therapeutic effectiveness of a given plasma level of the drugs.   Information on the binding of these compounds is also of practical importance in interpreting the absorption rates of the drugs from the rates of increase of the blood concentration and in the measurement of clearance values of the compounds.

METHODS.   Studies were performed on fresh human blood from several normal subjects, using oxalate mixture as anticoagulant.   The plasma dialysis experiments were conducted as described by Davis (4).   The buffer salt solution was adjusted to pH 7.4 and the plasma in cellophane bags was dialyzed against this solution for 48 hours in the refrigerator.   The protein concentrations of the plasma samples after dialyzing were measured as described by Kingsley (8).   Sulfonamide concentrations were measured according to Bratton and Marshall (9); when known amounts of the compounds studied were added to whole blood, the results of analysis accorded satisfactorily with the amounts added.

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[1] Throughout this paper, "acetyl" derivative refers to the N⁴-acetyl derivative.   All of these compounds were kindly supplied by Lederle Laboratories, Inc.

Calculations of the percentage of bound sulfonamide in the plasma were made as follows Plasma water per unit of dialyzed plasma (correcting by the formula used by Davis (4) for the small space occupied by protein) when multiplied by the concentration of the sulfonamide in the dialysate gives the concentration of unbound sulfonamide in the dialyzed plasma This value is subtracted from the total concentration of sulfonamide in the dialyzed plasma to give the concentration of bound drug Since some dilution of the plasma protein occurs during dialysis, the extent of binding for plasma of 7 0 gm of protein per 100 cc (i e , the average value for normal undialyzed plasma) is calculated by dividing the concentration of bound drug in the dialyzed plasma by the protein concentration (grams per 100 cc) and multiplying by 7 0 This last value is then divided by this last value plus the plasma concentration of unbound drug (as calculated above) and multiplied by 100 to give the percentage of bound sulfonamide (table 1)

The distribution of the sulfonamides between red cells and plasma was studied essentially as described by Heinemann (5) Solutions of the compounds were made in M/100 phosphate buffer with final pH of 7 4 and 1 or 2 cc of these solutions sufficient to give the desired concentrations of sulfonamide in the blood samples were dried in small flasks The sulfonamide was redissolved in the plasma of 5 or 10 cc of blood and the red cells then added The blood was shaken occasionally at room temperature for one hour, the blood with added sulfonamide was at pH 7 4-7 5 The data were calculated as milligrams of compound per 100 cc of cell and plasma water, using the values 72 and 94 per cent respectively for their water contents (10)

The percentages of the drugs bound in the plasma *in vivo* were calculated from the data of several investigators for the absorption, excretion and blood or plasma levels following single oral doses of the drug as follows [2] The average value for the subjects studied by each author was used The amount of drug excreted in the urine during the first 8 or 12 hour period (corresponding with the time that blood level and urinary excretion data were available) after the drug was ingested was subtracted from the total amount excreted in the urine the value obtained being taken as the total amount in the body (A) (table 4) The concentration of the drug to be expected in the body water (B) if the drug were equally distributed throughout the body was calculated by dividing the amount in the body (A) by the water of the body The concentration of dialyzable drug in the plasma (C) was estimated by multiplying B by 94/100 (to provide a small correction for the space taken by the protein in the plasma) When C is subtracted from the total plasma concentration of sulfonamide (D) and the result divided by D and multiplied by 100 an estimate of the per cent of the drug bound in the plasma (E) is obtained

Since the concentration of total drug (bound + unbound) in the blood is higher than the calculated concentration of dialyzable drug (B) the value D which was gained by assuming at first (for purposes of this analysis) that the drug was equally distributed through out the body is obviously slightly too high When B is corrected by taking into account this recognized unequal distribution due to binding in the blood the newly calculated per cent of bound sulfonamide in the plasma becomes increased by 3 (±1) (i e , 49 per cent bound = 52 per cent bound) As this correction is small and certain further assumptions were necessary to make it, the values presented for per cent of bound sulfonamide (E)

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[2] Certain assumptions were made in these calculations The average weight of the adult subjects studied was considered to be 70 kilograms if the weight was not stated, and the water content of the body was taken as 75 per cent of the body weight The total amount of drug excreted in the urine during the several days after the drug was administered orally was considered to represent the amount absorbed and absorption was considered complete in 8 to 12 hours after ingestion of these drugs The calculations are made on the basis of total drug in the body (i e free plus acetylated), the error introduced is small since the binding in the plasma of the acetylated compounds does not differ greatly from that of the parent drugs When the plasma sulfonamide level was not given, it was estimated by multiplying the blood level by the factor 1 4 (5 7) (table 3)

in table 4 as gained from the data of different investigators are the uncorrected values; the average values for each drug include the correction as indicated (table 4).

RESULTS. The *in vitro* dialysis studies (table 1) show that sulfamerazine and sulfamethazine are bound in normal human plasma to a considerably greater extent than is sulfadiazine. Similarly, acetylsulfamerazine and acetylsulfamethazine are bound to a greater extent than is acetylsulfadiazine; at a given plasma concentration of compound, all of these acetyl derivatives appear to be bound to a somewhat greater extent than are the respective drugs (table 1). As the plasma concentration of drug is decreased, the percentage of binding of the drug increases significantly (table 1). The results for different individuals agree well; some variation is to be expected in accord with the slight normal

TABLE 1

The degree of binding of 2-sulfanilamidopyrimidine compounds in normal human plasma at pH 7.4

(Calculated for plasma protein concentration of 7.0 gm. per cent)

SUBJECT	COMPOUND					
	Sulfadiazine		Sulfamerazine		Sulfamethazine	
	Plasma level	Amount of drug bound	Plasma level	Amount of drug bound	Plasma level	Amount of drug bound
	mg./100 cc.	*per cent*	*mg./100 cc.*	*per cent*	*mg./100 cc.*	*per cent*
B. B..... ..	18.8	54	27.8	70	28.2	71
G. R.—1....	15.2	52	23.1	71	23.8	72
A. T.... ..	9.3	57	17.7	84	16.1	85
M. V.......	8.8	54	11.9	82	9.2	78
G. R.—2....	4.4	60	8.2	87		
	Acetylsulfadiazine		Acetylsulfamerazine		Acetylsulfamethazine	
A. G..... .	26.2	60	27.6	78	36.4	78
G. R.—2....	14.8	65	18.9	88	23.8	88
M. V.......	12.5	58	18.9	84	25.4	84

variations in plasma albumin:globulin ratios. For a plasma level of drug of 10 mgm. per 100 cc. and protein concentration of 7.0 gm. per 100 cc., the average values for binding are estimated as 56 per cent for sulfadiazine, and 84 per cent for both sulfamerazine and sulfamethazine.

Expressing the findings in terms of dialyzable sulfonamide in the plasma (table 2), the data show that blood with 20 mgm. per 100 cc. of sulfamerazine or sulfamethazine contains approximately the same molar concentration of freely diffusible drug in the plasma as does blood with 10 mgm. per 100 cc. of sulfadiazine.

The distribution ratios of the three drugs between the red cells and plasma were very variable (table 3). The concentrations of all three drugs were lower in the water of the cells than in that of the plasma. The cell:plasma ratios of the acetyl derivatives of the drugs were consistently lower than the ratios for the free drugs (table 3).

Calculations from available data on absorption, excretion and blood levels following single doses of these drugs gave average values for *in vivo* binding of 52 per cent of the plasma sulfadiazine compared with 77 per cent for sulfamerazine

TABLE 2

Comparative concentrations of dialyzable (unbound) drugs in plasma calculated for blood concentrations of drug of 10 and 20 mg per cent

COMPOUND	DRUG IN WHOLE BLOOD	DRUG IN PLASMA	CONCENTRATION OF DIALYZABLE DRUG IN PLASMA H₂O	
	mg /100 cc	mg /100 cc	mg /100 cc	$M \times 10^{-4}$
Sulfadiazine	10	14	6 16	2 46
Sulfamerazine	10	14	2 24	0 85
Sulfamethazine	10	14	2 24	0 81
Sulfadiazine	20	28	14 0	5 60
Sulfamerazine	20	28	8 12	3 07
Sulfamethazine	20	28	8 12	2 91

* Calculated from date of table 1, using factor of 1 4 for estimating plasma levels from whole blood levels The concentration of dialyzable drug in the plasma water at plasma levels of 14 mg per cent was taken as 44 per cent of the total plasma concentration for sulfadiazine and 16 per cent for sulfamerazine and sulfamethazine, at plasma levels of 28 mg per cent the values taken were 50 per cent for sulfadiazine and 29 per cent for sulfamerazine and sulfamethazine

TABLE 3

In vitro cell plasma ratios of 2 sulfanilamidopyrimidine compounds in normal human blood

SUBJECT	COMPOUNDS		
	Sulfadiazine Cell plasma rat o × 100	Sulfamerazine Cell plasma rat o × 100	Sulfamethazine Cell plasma rat o × 100
A T	50	20	26
B B	52	93	70
A G	70	32	33
G R —1	89	106	107
	Acetylsulfadiazine	Acetylsulfamerazine	Acetylsulfamethazine
A G	38	2	7
G R —2	27	5	15
M V	27	18	0

* The whole blood levels of drugs varied from 8 to 12 mg per cent, the whole blood levels of the acetyl derivatives from 6 to 10 mg per cent

and 73 per cent for sulfamethazine (table 4) These values correspond within reasonable expectation with those found in the *in vitro* dialysis studies (table 1)

DISCUSSION The extent of binding of sulfadiazine in the plasma in these experiments accords well with the results of Davis (3, 4, 11) from similar dialysis experiments, and of Heinemann (5) from ultrafiltration studies Davis (3, 4)

has also previously observed for other sulfonamide compounds that the extent of binding increases significantly as the concentration of drug in the plasma decreases. Sulfamerazine and sulfamethazine are bound to a somewhat greater extent (table 1) than is sulfathiazole (3, 5), this latter compound being the most highly bound (75 per cent (3)) of any of the other sulfonamide drugs for which binding has been reported. From the results of electrophoresis studies of protein fractions of human serum, it has been concluded that the sulfonamide drugs are bound chiefly to plasma albumin (4).

TABLE 4

Calculated percentages of bound sulfonamides in plasma in vivo

(Calculated from data on absorption, excretion, and blood levels in man after single oral doses of the drugs)

DRUG	REFERENCE TO DATA	NO OF SUBJECTS AVERAGED	A AMT. OF DRUG IN BODY 8-12 HRS AFTER DOSE	B CON OF DRUG IN BODY WATER IF DISTRIBUTED EQUALLY	D CON OF DRUG IN PLASMA 8-12 HRS AFTER DOSE	E CALCULATED % BOUND IN PLASMA
			grams	mg /100 cc	mg /100 cc	
Sulfadiazine	(1)	3	2.8	5.3	10.0	50
Sulfadiazine	(6)	9	1.7	3.2	6.0	50
Sulfadiazine	(2)	14	1.7	3.2	5.5	46
Av. + 3						52*
Sulfamerazine	(1)	3	3.3	6.3	24.5	76
Sulfamerazine	(2)	14	2.2	4.1	13.3	71
Sulfamerazine	(7)	8	1.8	3.4	10.5	70
Sulfamerazine	(7)	2†	2.8	5.2	20.9	77
Av. + 3			.			77
Sulfamethazine...	(2)	14	1.8	3.4	10.5	70
Value + 3 .						73

* The value of 3 is added to the averages in accord with the considerations discussed in section on Methods.

† Intravenous sodium sulfamerizine was injected in these 2 subjects. The plasma value $\frac{1}{2}$ hour after injection was used; extretion in $\frac{1}{2}$ hour was considered negligible.

From data available earlier for sulfanilamide, sulfapyridine, sulfadiazine and sulfathiazole, it was suggested (11) that the percentage of binding at pH 7.4 of the sulfonamides appeared to vary directly with the percentage of acid ionization at this pH. Analysis of the larger amount of data now available for binding and for the acid dissociation (12) of sulfonamide drugs, however, shows no such correlation (table 5). It is to be noted particularly that although sulfanilamide is essentially unionized and sulfacetimide is practically completely ionized at pH 7.4, both drugs are bound to approximately the same extent, 20 per cent (table 5).

The large variability in the cell:plasma ratios of sulfadiazine, sulfamerazine and sulfamethazine found in the *in vitro* studies reported here accord with the

variability found by others for blood drawn from patients receiving these drugs (1, 6, 7) and in previous *in vitro* studies of sulfadiazine (5). This variability is so great as to raise the question of whether all the necessary factors are controlled in the course of studying the drawn blood. Since these drugs were often (table 3) found in the cells in a concentration higher than the concentration of the unbound drug in the plasma, the data do not support the suggestion of others that the relative amounts of sulfonamide drugs in the cells is determined by the dialyzable fraction of the drug in the plasma (5). The cell:plasma ratios of the acetyl derivatives of these drugs are generally lower than for the parent drugs and appear to vary less widely in different blood samples[3] (table 3). Lower cell:plasma ratios for acetylsulfapyridine than for sulfapyridine have also been

TABLE 5

Lack of correlation between the extent of binding of sulfonamide drugs in plasma at pH 7.4, and the percentage of ionization at this pH

DRUG	pKa[*]	PER CENT ACID IONIZATION AT pH 7 4	APPROX PER CENT BOUND AT pH 7 4[†]
Sulfaguanidine		0.0	25
Sulfanilamide	10.43	0 1	20
Sulfapyridine	8.43	8 7	40
Sulfamethazine	7 37	52.0	84
Sulfathiazole	7.12	65 5	75
Sulfamerazine	7 06	68.5	84
Sulfadiazine	6 48	89 3	56
Sulfapyrazine	6.04	96 0	50
Sulfacetimide	5.38	99 0	20

[*] pKa values from Bell and Roblin (12).

[†] For concentrations of 7 gm per cent plasma protein and 10 mg per cent plasma sulfonamide.

The values for percentage of drugs bound are from the data of this paper (table 1), Davis (3), and calculated from data of Davis and Wood (11).

reported (13). This problem of the cell:plasma ratios of sulfonamide compounds requires further clarification.

The close correspondence between the values for the percentage of binding of these drugs in the plasma as found by the *in vitro* dialysis experiments and as estimated *in vivo* from pharmacological data appears to attest to the validity of the latter estimations. This correspondence also suggests that no appreciable amounts of these sulfonamides are bound in the tissues, i e that the concentration of the drug in the tissues is the same as the concentration of the dialyzable portion of the drug in the plasma. The calculations are obviously too gross to give infor-

[3] When the data for acetylsulfamerazine and acetylsulfamethazine (table 3) are recalculated after correcting the hematocrit for plasma trapped in the cell mass (20), the results indicate that neither of these compounds enters the red cells However, preliminary data on the ratios of the concentrations of these compounds in the red cells and isotonic, buffered saline solutions of pH 7 4 have shown the unbound compounds present in the cells in approximately the same concentrations as in the saline solutions

mation concerning the distribution of the drug between plasma and any small selected portion of the body, such as the brain or cerebrospinal fluid. Similar calculations from available data for sulfanilamide, sulfathiazole and sulfapyridine also show a good correpondence between the estimated binding *in vivo* and the results of others (3, 5) for *in vitro* binding.

It has been pointed out previously (4) that the distributions of sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine between blood and cerebrospinal fluid are very similar to the calculated distributions for these drugs between blood and its dialysate.[4] Similarly, the data of Murphy et al. (7) for the distribution of sulfamerazine between plasma and cerebrospinal fluid after oral administration of this drug are very similar to the *in vitro* distribution findings between plasma and its dialysate (table 1). Pleural and ascitic fluid : plasma ratios for sulfamerazine (7) also accord with those expected for dialysates containing appreciable amounts of protein. Rose et al. (16) found a higher average ratio for cerebrospinal fluid:blood concentration of sulfamethazine than the calculated (from data of table 1) dialysate:blood ratio, and also found the average sulfamethazine concentration of 3 pleural fluid specimens to be about 10 per cent higher than that of the compared blood specimens.

As shown in table 3, blood levels of approximately 20 mgm. per 100 cc. for sulfamerazine and sulfamethazine provide approximately the same concentration of dialyzable drug as a blood level of only one-half this amount of sulfadiazine. With sulfonamide drugs which are bound in the plasma to different degrees, comparisons of absorption rates after oral administration on the basis of relative rates of increase of blood or plasma sulfonamide levels must be made with caution. For a given amount of drug in the body the plasma levels will be higher for the drugs which are bound to the greater extents. Thus when approximately the same amounts of sulfadiazine, sulfamethazine or sulfamerazine have been absorbed into the body, one would expect the blood or plasma levels of the latter two drugs to be approximately two to three times as great as that of sulfadiazine. Although other investigators (2) have concluded from the more rapid rate of increase of the blood levels after single oral doses of sulfamerazine and sulfamethazine that these drugs are more rapidly absorbed than is sulfadiazine, when the data are calculated on the basis of the amount of unbound, diffusible drug present throughout the body water at a given blood level for each drug it is concluded that the rates of absorption of these three drugs are actually very similar.

It is of therapeutic interest that Davis and Wood (11) have reported from *in vitro* experiments with other sulfonamides that the bound sulfonamide in the plasma is probably bacteriostatically ineffective. The great variation in the degree of binding of the sulfonamides in the plasma again raises the question to the clinical investigator as to what is the optimum therapeutic blood level of a particular sulfonamide. The tendency of investigators who are currently subjecting sulfamerazine and sulfamethazine to clinical trials is to give daily doses of these

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[4] It must be noted that cerebrospinal fluid is not a true dialysate of plasma (14, 15) and that these correspondences may be fortuitous and may not be applicable to all sulfonamide compounds.

drugs (i. e., 3 to 4 gm. of sulfamerazine (7, 17), and 6 to 8 gm. or more of sulfamethazine (16, 18, 19)) to maintain approximately the same free (i.e., not acetylated) blood levels of these sulfonamides as are maintained by 6 gm. daily of sulfadiazine. Inasmuch as the *in vitro* quantitative bacteriostatic activities of these three drugs (unbound) appear to be similar (12) and the plasma and tissue concentrations of unbound drug are much lower for sulfamerazine and sulfamethazine than for sulfadiazine at any given plasma level of total drug (bound + unbound), the results of carefully controlled comparative clinical trials will be of theoretical as well as practical interest. From these considerations it appears not unlikely that the therapeutic effectiveness of a given blood or plasma level (bound + unbound drug) of sulfamerazine or sulfamethazine would be only about one-third to one-half as great as the same level of sulfadiazine, and that on the average the clinical response obtained with a given blood level of these newer drugs might not be as satisfactory as obtained with the same blood level of sulfadiazine.

SUMMARY

1. *In vitro* dialysis studies show sulfamerazine and sulfamethazine to be bound in the plasma to a much greater extent than is sulfadiazine. Similarly, their acetyl derivatives are more highly bound in the plasma than is acetylsulfadiazine.

2. At a drug concentration of 10 mgm. per 100 cc. in plasma of pH 7.4 and containing 7.0 gm. per 100 cc. of protein, only 16 per cent of the plasma sulfamerazine and sulfamethazine are freely diffusible as compared with 44 per cent for sulfadiazine.

3. Estimations of the extent of binding *in vivo*, made from available data on absorption, excretion and blood levels in man after single doses of these three drugs, afford evidence that the binding of the drugs in the plasma *in vivo* is essentially the same as was found in the *in vitro* studies.

4. It is calculated that a blood level of 20 mgm. per 100 cc. of sulfamerazine or of sulfamethazine provides a concentration of dialyzable drug in the plasma and tissues approximately the same as is provided by a blood level of only one-half this amount of sulfadiazine.

5. The results of *in vitro* studies of red cell:plasma ratios of all these drugs were very variable. The red cell:plasma ratios of the acetyl derivatives of the drugs were generally lower than those of the parent drugs.

6. The relative extent of binding of these drugs in the plasma is discussed with respect to the interpretation of absorption rates from the rate of increase of the blood concentration of the drugs and with respect to its possible bearing on the relative therapeutic effectiveness of a given blood level of the different drugs.

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COMPARISON OF THE PRESSOR ACTION OF SOME OPTICAL ISOMERS OF SYMPATHOMIMETIC AMINES

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It is a well known fact that the optical rotation of organic compounds has a significant influence upon their pharmacologic action. Several excellent examples have been given by Cushny in his Dohme lectures (1). In many instances, the levo and the dextrorotatory isomers possess qualitatively the same type of action, but the *l* isomer is considerably more powerful than the *d* form. Cushny (2) himself demonstrated that *l* epinephrine was 12–15 times as strong as the *d* isomer on the sympathetic endings of the vessels. Of the optical isomers of ephedrine, Chen, Wu, and Henriksen (3) found that *l* ephedrine was about 3 times as active as *d* ephedrine, and *d* ψ ephedrine 7 times as active as *l* ψ ephedrine, in raising blood pressure of pithed cats. Tainter and Stockton (4) showed that 'Neo synephrine' (*l* α Hydroxy β methylamino-3 hydroxy ethylbenzene, Stearns) had a greater pressor action than its *d* isomer in atropinized cats. Schaumann (5) reported that the ratio of pressor activity of *l* and *d* 3,4 dioxy norephedrines was 160 : 1. Similarly, Alles and Knoefel (6) found that in rabbits *l* β hydroxypheno methylamine was 5–10 times more active as a pressor agent than the *d* isomer. The response of the isolated rabbit's uterus and small intestine to 3,4 dioxy *nor* ephedrine and ephedrine is similar to the changes in blood pressure (5, 7)—the *l* isomer being more powerful in each instance. Alles (8) claimed that the *l*, *dl*, and *d* forms of benzedrine (Amphetamine, S K F) induced pressor effects of equal intensity in anesthetized dogs and rabbits by intravenous injection, and in man by mouth. The lack of differences among the 3 isomers would constitute a unique exception to the group of closely related products enumerated above.

Of particular interest is the reversed order of activity of the optical isomers of benzedrine upon the central nervous system. That *d* benzedrine had a greater stimulating action than the *l* isomer was first observed by Alles (8), and substantiated by Prinzmetal and Alles (9), Schulte, Reif, Bacher, Lawrence, and Tainter (10), and Novelli and Tainter (11). Morrison and Abreu (12) observed that *d* benzedrine increased the oxygen uptake of dogs' brains depressed by morphine more than its *l*-enantiomorphic isomer. Knoefel (13) concluded that the *d* isomer of either benzedrine or desoxyephedrine was more active than the *l* isomer in increasing the work output as measured by a bicycle ergometer.

With a total of 18 optical isomers of sympathomimetic amines available in this laboratory, an excellent opportunity was afforded to compare their pharmacologic action with relation to their optical rotation. Ten diastereoisomeric salts of 4 aromatic amines were generously supplied by Professor Walter H. Hartung, School of Pharmacy, University of Maryland, Baltimore—*l* and *d* propadrine

l-mandelates, *l*- and *d*-ψ-propadrine *l*-mandelates, *l*- and *d*-benzedrine *l*-mandelates, *l*-benzedrine *d*-mandelate, *l*- and *d*-isobenzedrine [I] *l*-mandelates, and *d*-isobenzedrine *d*-mandelate. Dr. E. H. Volwiler of Abbott Laboratories, North Chicago, favored us with *l*-, *dl*-, and *d*-desoxyephedrine [II] hydrochlorides; and Dr. William E. Kirsch of Smith, Kline and French Laboratories, with *l*-, *dl*-, and *d*-benzedrine sulfates.

TABLE 1
Comparison of pressor action of stereoisomers of epinephrine as bitartrate

CAT NUMBER	SEX	WEIGHT	DOSES PRODUCING EQUAL PRESSOR RESPONSES		RATIO OF ACTIVITY *l/d*
			l-ISOMER	*d*-ISOMER	
		kg.	*μg. (total)*	*μg. (total)*	
1	F	2.415	16.67	400	24.0
2	F	2.965	16.67	250	15.0
3	F	2.340	16.67	300	18.0
4	F	2.200	16.67	300	18.0
5	F	2.370	16.67	300	18.0
6	F	2.620	16.67	350	21.0
7	M	2.600	16.67	300	18.0
8	F	3.120	16.67	300	18.0
9	F	2.100	22.22	550	24.8
10	F	2.485	27.50	450	16.4
11	M	2.180	20.00	400	20.0
12	M	3.360	25.00	400	16.0
13	F	2.430	20.00	400	20.0
14	F	2.455	16.00	300	18.8
15	M	2.300	10.00	200	20.0
16	M	2.995	10.00	200	20.0
17	M	2.935	12.00	160	13.3
18	F	3.660	4.80	80	16.7

Geometric mean ± S. E., 18.46 ± 0.66.

$$C_6H_5 \cdot CH \cdot CH_2 \cdot NH_2 \qquad C_6H_5 \cdot CH \cdot CH \cdot NHCH_3$$
$$\underset{CH_3}{|} \qquad\qquad\qquad \underset{CH_3}{|}$$
$$[I] \qquad\qquad\qquad\qquad [II]$$
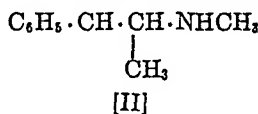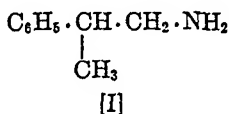
The *l*- and *d*- isomers of epinephrine in form of bitartrates were purchased in Germany prior to the start of World War II.

METHOD. Evaluation of the pressor action of epinephrine was accomplished in pithed cats according to Elliott's procedure (14). Since there is no loss of response by repeated intravenous injections, the *l*- and *d*-isomers were matched in the same animals until equivalent doses reproduced an identical rise of blood pressure.

In the midst of our work, the cat method of digitalis assay in U.S.P.XII became official. Thus, there was a sudden shortage of cats. A few preliminary experiments indicated that the dog would be equally satisfactory after decerebration and pithing. The remaining 16 compounds were therefore tested out in the dog. As in the case of ephedrine (15), no two

enantiomorphic substances could be compared in the same animal because whichever was injected first depreciated the value of the other   As an alternative, each isomer was compared against variant doses of epinephrine for the equivalent intensity of pressor action   The ratio could then be calculated from the equivalent doses of epinephrine   A minimum of 3 to 9 dogs was employed for each product

RESULTS   The data obtained in 18 cats with l and d epinephrine bitartrates are summarized in table 1   As indicated in the last column, the mean (geo

TABLE 2

Comparison of pressor action of optical isomers of aromatic amines related to ephedrine

OPTICAL ISOMER OF AMINE	NUMBER OF DOGS USED	RANGE OF DOSES STUDIED	GEOMETRICAL MEAN OF EPINEPHRINE EQUIVALENT TO 1 MG OF AMINE IN RAISING BLOOD PRESSURE	RATIO OF ACTIVITY ± STANDARD ERROR
		mg (total)	*µg*	
l Desoxyephedrine hydrochloride	4	5–20	1 80	1 0
dl Desoxyephedrine hydrochloride	9	5–15	1 44	0 799 ± 0 146
d Desoxyephedrine hydrochloride	5	10–48	1 27	0 707 ± 0 128
l Benzedrine sulfate	3	3–4	5 31	1 0
dl Benzedrine sulfate	5	4–6	4 48	0 842 ± 0 118
d Benzedrine sulfate	8	2 S	3 7S	0 712 ± 0 08
l Benzedrine *l* mandelate	4	5–7 5	3 54	0 969 ± 0 08
l Benzedrine *d* mandelate	3	5–7 5	2 40	0 656 ± 0 063
d Benzedrine *l* mandelate	5	4–10	3 66	1 0
l Isobenzedrine *l* mandelate	5	5–10	2 0S	1 0
d Isobenzedrine *l* mandelate	5	5–10	1 77	0 850 ± 0 175
d Isobenzedrine *d* mandelate	5	10–14	1 41	0 677 ± 0 128
l Propadrine *l* mandelate	5	5–7 5	3 06	1 0
d Propadrine *l* mandelate	5	5–15	2 0S	0 678 ± 0 103
l ψ Propadrine *l* mandelate	5	10–20	0 57	1 0
d ψ Propadrine *l* mandelate	5	10–20	0 50	0 869 ± 0 183

metric) ratio of pressor activity between l and d isomers is about 18 5 1   This figure approximates the range of 15–20 as reported by Abderhalden (16), Tiffeneau (17), Tainter (18), and Nathanson (19), but is at variance with the conclusions of Tromherz (20)

The results with the other compounds are given in table 2   Without exception, the l isomer is more powerful in raising blood pressure of pithed dogs than the d isomer   Thus, d desoxyephedrine and d benzedrine sulfate have approximately 71% of the activity of their l forms   Their racemic mixtures, as expected,

give rise to values lying between those of the *l*- and *d*-isomers. Among the diastereoisomers, namely, the optically active salts of the optically active amines, the *l*-mandelates of *l*-isobenzedrine, *l*-propadrine, and *l*-ψ-propadrine are all more active than the *l*-mandelates of their *d*-isomers. Examples of experiments with propadrine are illustrated in figure 1.

The most remarkable feature of the diastereoisomeric salts is the relatively higher activity of the *l*-mandelates. Thus, *l*-benzedrine *l*-mandelate is about
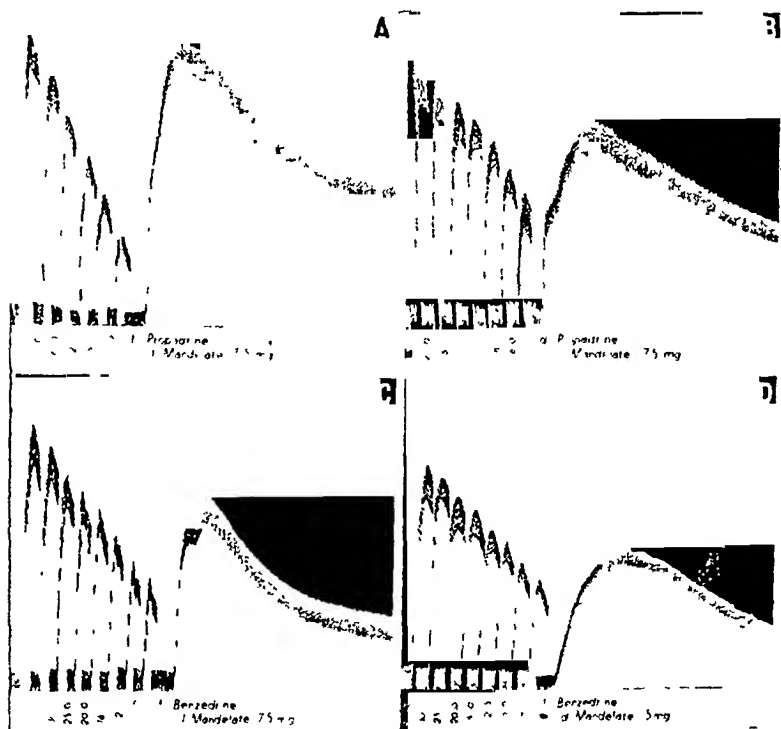
FIG. 1. COMPARISON OF PRESSOR ACTION IN PITHED DOGS

A, B, C, and D designaté results from 4 different dogs   All the numbers on the left-hand side of each tracing indicate the doses of epinephrine in μg. All injections were made intravenously.

⅓ stronger than *l*-benzedrine *d*-mandelate. Examples are given in figure 1. Similarly, *d*-isobenzedrine *l*-mandelate is more potent than *d*-isobenzedrine *d*-mandelate. The relatively high activity of *d*-benzedrine *l*-mandelate, comparable to that of *l*-benzedrine *l*-mandelate, is also suggestive of the favorable influence of *l*-mandelic acid in the molecule. In this connection, it is appropriate to mention Cushny's work on tropeines (21). *l*-Homatropine, or *l*-mandelic acid ester of tropine, is approximately twice as active as *d*-homatropine which is *d*-mandelic acid ester of tropine. Equally interesting is the recent publication

of Auhagen (22), in which he showed that while p aminobenzoyl l glutamic acid is 8–10 times stronger than an equimolecular quantity of p aminobenzoic acid in antagonizing sulfanilamide against *Streptobacterium plantarum*, the p aminobenzoic acid derivative of d glutamic acid is inactive

SUMMARY

1 The pressor activity of d- and l epinephrine bitartrates has been compared in pithed cats The ratio of the d- to the l isomer is approximately 1 18 5

2 The pressor activity of optical isomers of desoxy ephedrine HCl, benzedrine sulfate, isobenzedrine l mandelate, propadrine l mandelate, and ψ propadrine l-mandelate has been evaluated in pithed dogs In every instance the l isomer is more active than the d isomer The racemic mixtures of desoxyephedrine HCl and benzedrine sulfate are intermediate in action between their l and d isomers

3 Of the diastereoisomeric salts, namely, the optically active mandelates of the optically active amines, the l mandelate has a relatively higher pressor activity as compared with the d mandelate Thus, l benzedrine l-mandelate is more powerful than l benzedrine d mandelate, and d isobenzedrine l mandelate, more powerful than d isobenzedrine d mandelate

Acknowledgment The authors are indebted to Mr John C Hanson for his assistance in compiling and analyzing the results of this investigation

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THE PHARMACOLOGICAL ACTION OF N-METHYLCYTISINE

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N-Methylcytisine has been known for years to occur in a number of plants. The chemical properties of the compound have been repeatedly studied. It was first prepared chemically by Partheil (1). In 1913, Power and Salway (2) showed that the alkaloid of *Caulophyllum thalictroides* was methylcytisine. The substance had been previously extracted from the same species by Lloyd (3) in 1893 and named caulophylline, but its true chemical identity was not determined at that time. Several members of Papilionaceae have since been found to contain methylcytisine (4). Investigation of the pharmacological action of this alkaloid has been casual, however. Power and Salway mentioned that Laidlaw had tested their preparation and had demonstrated an action similar to cytisine, but one-tenth as active. Further work is limited to a toxicity determination in mice (5). Consequently, additional study of methylcytisine appeared desirable. The compound was generously supplied to us by Dr. Richard H. F. Manske, Division of Chemistry, National Research Council, Ottawa, Canada, who isolated it from *Thermopsis rhombifolia* (6).

Methylcytisine is a colorless substance occurring in prismatic crystals. It is readily soluble in water, alcohol, chloroform, or benzene. The melting point is 138°C. According to Späth and Galinovsky (4), it has the following structural formula:
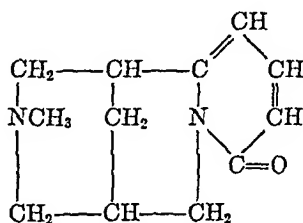
EXPERIMENTAL. *Action on frog heart ganglia.* The intact frog's heart was perfused according to the method of Howell and Cooke (7) using a Greene cannula. The threshold current necessary to produce complete cardiac inhibition by stimulation of the right vagosympathetic nerve was determined. Then methylcytisine solution was applied directly to the heart by a medicine dropper. The drug itself caused cardiac standstill within 30 seconds, but normal rhythm and contractility were reëstablished 5 minutes later. Using the threshold current the vagus was again stimulated but inhibition did not occur. However, stimulation of the white crescent (sinus region) resulted in typical inhibition. This procedure was repeated with nicotine. Both sub-

stances produced similar actions Nicotine was roughly 25 times as potent as methylcytisine, however, and its action was more rapid

Circulatory action in dogs Under ether anesthesia, femoral blood pressure was recorded with a mercury manometer The right vagus nerve was isolated for stimulation by an inductorium After determining the minimal current necessary to produce temporary cardiac arrest, methylcytisine was injected intravenously When the blood pressure had returned to the original level, the vagus nerve was again stimulated If cardio inhibition did not result, pilocarpine was given intravenously A similar test was made with nicotine
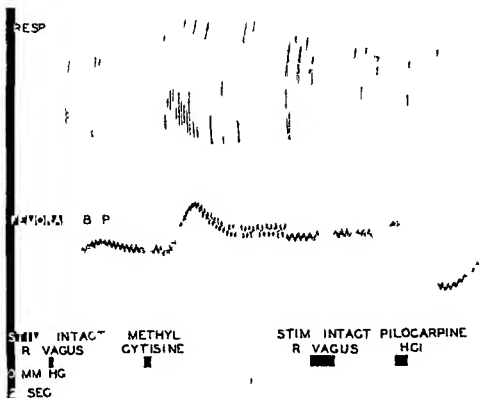
FIG 1 THE ACTION OF METHYLCYTISINE ON THE CIRCULATION OF ETHERIZED DOGS
Blood pressure and respiration were recorded in the usual manner The doses were
intravenous injection
by failure of vagus in

A record of the action of methylcytisine is shown in figure 1 The results with the 2 drugs were the same Both produced a temporary rise of blood pressure followed by loss of inhibitory effect of vagus stimulation That the post ganglionic nerve endings were not paralyzed was demonstrated by the cardio inhibitory effect of pilocarpine In dogs, methylcytisine appeared to be about one tenth as active as nicotine

Respiratory stimulation through the carotid sinus As demonstrated by Heymans et al (8), small doses of nicotine stimulate respiration reflexly through the carotid sinus A similar action was shown for cytisine by Anitschkow (9) Methylcytisine was tested for this action in dogs under ether anesthesia Both carotid sinus nerves were isolated according to the technic of Schmidt (10)

Methylcytisine was then given intravenously. It produced respiratory stimulation. After both sinus nerves were cut, the same dose of the drug was repeated. Only a slight increase of respiration occurred, probably from stimulation of the respiratory center. There was a rise of blood pressure equal to that from the first dose, however. Nicotine gave parallel results with this same procedure. A record of the action of methylcytisine is seen in figure 2. Here again, nicotine was about 10 times as active as methylcytisine.

Effect on isolated rabbit's small intestine. An attempt was made to prove the nicotine-like action of methylcytisine on the intrinsic ganglia of the rabbit's small intestine. The following procedure was employed. A record of contrac-
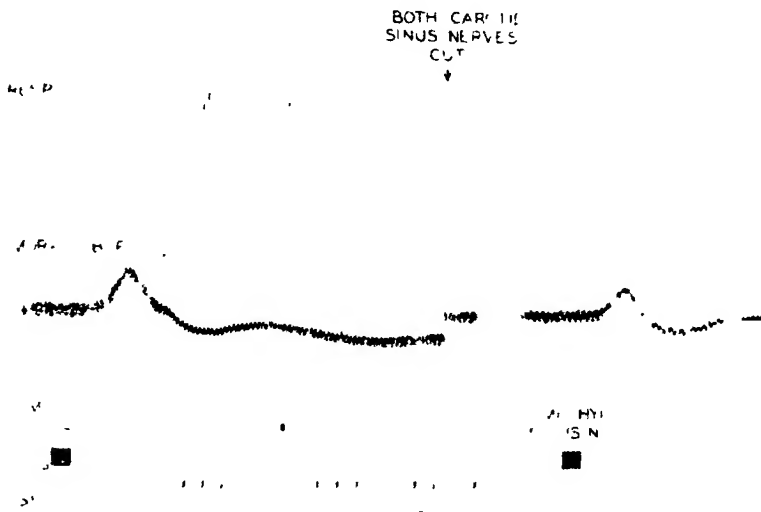
FIG. 2. THE RESPIRATORY STIMULATING ACTION OF METHYLCYTISINE

Under ether anesthesia the dog's carotid sinus nerves were isolated. A 50-mg. dose of methylcytisine was injected intravenously before and again after cutting both sinus nerves. The respiratory and blood pressure responses to the drug were decreased as the result of sinus denervation.

tions of the isolated small intestine of a rabbit was obtained by suspension of a segment in an aerated bath of Ringer-Locke's solution at a constant temperature of 38°C. Repeated doses of methylcytisine were given until response to the drug failed to occur. Then, successively, pilocarpine, epinephrine, and barium chloride were added to the bath without changing the solution. The whole was finally washed out and replaced by a fresh supply of Ringer-Locke's solution. The demonstration of typical pilocarpine, epinephrine, and barium actions when methylcytisine no longer stimulated was evidence that the nerve endings and muscle cells were functioning. The ganglion cells, however, appeared to be paralyzed. For comparison, nicotine was also tested by this procedure with identical findings. Graphic results are shown in figure 3. The activity of methylcytisine was about one-tenth as great as that of nicotine.

Hyperglycemic action in rabbits It is known that nicotine causes an elevation of the blood sugar (11), the rise being caused, in part at least, by stimulation of epinephrine secretion Tests were run on unanesthetized albino rabbits the marginal ear vein being used for taking blood samples and making injections
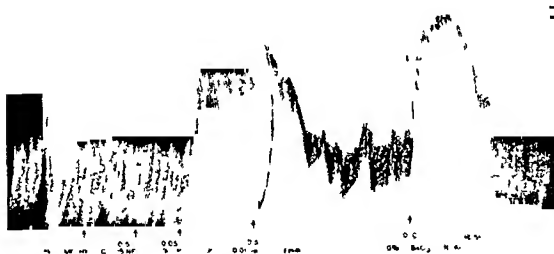
FIG 3 ACTION OF METHYLCYTISINE ON CONTRACTIONS OF THE ISOLATED RABBIT'S SMALL INTESTINE

A strip of the upper jejunum in aerated Ringer Locke's solution at 38°C was used the 100 cc bath without changing the solution After barium the bath was drained and filled
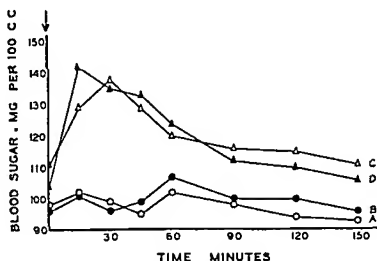
FIG 4 HYPERGLYCEMIA IN RABBITS FOLLOWING ADMINISTRATION OF METHYLCYTISINE Injections of the drug were made at zero time shown by the arrow The doses of methyl cytisine were 10 10 20 and 40 mg per kg for animals A B C and D respectively

Blood sugar determinations by the Miller Van Slyke method (12) were made at regular intervals for 2½ hours following injection of the drugs The doses for methylcytisine were 10, 20 and 40 mg per kg while only 1 mg per kg of nico tine was required This dose of nicotine, incidentally, proved to be convulsive

in the 2 animals tested.   No convulsions occurred with methylcytisine, although
the 40-mg. dose produced tremors.   The effects on blood sugar are graphically
shown in figure 4.   Doses of 10 mg. per kg. of methylcytisine had little or no
hyperglycemic action.   Nicotine in a dose of 1 mg. per kg. appeared to be about
equal to 20 mg. per kg. of methylcytisine.

Toxicity.   The median lethal dose ± standard error was determined by in-
jection into the tail vein of albino mice, weighing from 14 to 18 gm.   Computa-
tions were made according to the method of Bliss (13).   The results are seen in
table 1 along with those of nicotine determined for comparative purposes.
Nicotine proved to be nearly 40 times as toxic as methylcytisine in mice.   Fur-
ther, the reactions of the animals to these 2 compounds preceding death were not
identical.   Nicotine killed almost immediately or not at all, death being pre-

TABLE 1

The acute toxicity of N-methylcytisine and nicotine by intravenous injection into albino mice

ALKALOID	DOSE	NUMBER DIED / NUMBER USED	$LD_{50} \pm$ S E.
	mg. per kg		*mg. per kg.*
N-Methylcytisine	10.0	0/6	21.98 ± 0.90
	14.0	0/5	
	20.0	4/10	
	22.5	9/20	
	25.0	22/26	
	27.5	7/10	
	30.0	18/20	
	40.0	5/5	
Nicotine	0.250	0/5	0.579 ± 0.037
	0.350	0/5	
	0.365	2/10	
	0.400	2/10	
	0.500	8/20	
	0.700	11/20	
	1.000	15/15	

ceded by severe tonic convulsions.   Methylcytisine produced less severe con-
vulsions, at first clonic and then tonic in nature.   Death occurred from a few
minutes to a few hours after the injection.   The lethal dose of methylcytisine
in white mice as determined by Kalaschnikow and Kusnetzow (5) was 62.5 mg.
per kg. of body weight when given subcutaneously.   This is nearly 3 times our
value for the intravenous lethal dose.

DISCUSSION.   The above results show clearly that methylcytisine exerts a
pharmacological action on the nervous system closely resembling that of nico-
tine.   In the case of the parasympathetic ganglia, this consists of primary stimu-
lation followed by paralysis.   Nicotine, in addition, is known to act on the
sympathetic ganglia.   In this respect, methylcytisine by intravenous injection
into dogs produces a rise of blood pressure similar to that resulting from sym-
pathetic stimulation by nicotine.

Furthermore, methylcytisine apparently causes stimulation of the central

nervous system and then paralysis as shown by convulsions followed by prostration, cessation of respiration, and death   The compound is less active, however, than nicotine, because the convulsions are much less severe and the action occurs more slowly   The respiratory stimulation through the chemoreceptors of the carotid sinus is an additional point of similarity with nicotine No tests were made with methylcytisine for the curare-like action of nicotine

Methylcytisine is apparently weaker than cytisine   Dale and Laidlaw (14) compared the action of cytisine to that of nicotine and found it to be approximately as potent in cats, dogs, and rabbits   Since nicotine appears to be 10 to 40 times as strong as methylcytisine, the addition of the methyl group to cytisine obviously decreases its activity

CONCLUSIONS   The pharmacological action of N methylcytisine resembles that of nicotine and differs from it only in degree, as shown below

1   It stimulates and then paralyzes the ganglion cells of the cardiac vagus in frogs

2   In dogs it produces a rise of blood pressure followed by paralysis of the ganglia of the cardiac vagus

3   Respiration is reflexly stimulated through the chemoreceptors of the carotid sinus

4   On the isolated rabbit's small intestine, it first produces stimulation of motility   Subsequent doses fail to stimulate, but pilocarpine, epinephrine, and barium salts still cause their usual responses

5   When given intravenously to rabbits, it produces hyperglycemia

6   It is 10 to 40 times less active than nicotine in the various animals tested, its action occurs more slowly, and convulsions produced by it are less severe than those resulting from nicotine

7   Methylcytisine appears to be one fortieth as toxic as nicotine

The authors wish to express their thanks to Mr Harold M Worth for his technical assistance

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THE FATE OF D.B.E. ($\alpha\alpha$ DI-(p-ETHOXYPHENYL)β-PHENYL BROMO-ETHYLENE) IN THE BODY

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In a recent communication (Robson, Schönberg and Tadros, 1942) a new oestrogen, derived from triphenyl ethylene, was described. This compound has the property of producing a comparatively prolonged oestrogenic action when administered orally (i.e. by stomach tube) to mice, particularly when given in doses several times the amount necessary to produce a threshold response.

It appeared desirable to determine what happened to the compound following its administration, with special reference to any sites at which it might be stored. It is known that the natural oestrogens (oestradiol, oestrone) are rapidly inactivated in the body and this accounts for the relatively short duration of their action. Even when large doses are given only a small percentage can be recovered from the body tissues and from the excretions. There is also good evidence that the liver plays an important part in the destruction of the natural oestrogens (Zondek, 1934; Israel et al., 1937; Heller, 1940).

The fate of the synthetic oestrogen, stilboestrol, is somewhat different, in that an appreciable percentage of a dose given (about 20%) can be recovered from the urine; this oestrogen is, moreover, also rapidly eliminated from the body after absorption, though appreciable quantities are stored at the site of injection (Zondek and Sulman, 1939; Stroud, 1939).

METHODS. The experiments were performed on ovariectomized mice and on rabbits. They received doses of D.B.E. dissolved in oil of sesame by stomach tube. After various periods the animals were killed and the whole body or various organs extracted. In some experiments the urine and faeces were collected and also extracted.

The following method was used for the extraction of the oestrogen. The animals or tissues were minced and then ground in a glass mortar. The resulting mass was extracted with 10 times its weight of acetone at room temperature for 24 hours. The fluid was then decanted and the process repeated with fresh acetone for another 24 hours. The fluid was again decanted and the residue was extracted under a reflux condenser with six times its weight of boiling benzene for 6 hours. The benzene was then filtered off and added to the acetone extracts, and the mixed fluids were evaporated on a water bath. A current of air playing on the surface helped in the evaporation. The residue was used either as such or dissolved in oil of sesame.

The oestrogen content of the extracts was estimated by the intragastric administration of different amounts of the material to groups of ovariectomized mice. It has been found previously that doses of 10μgm of D.B.E., given orally in oil of sesame, produce an oestrogenic effect which falls to half in about seven days (when the vaginal effect is measured by the method of Robson, 1938), and the amount of material producing such an effect was assayed to contain 10μgm. of D.B.E.

The validity of the method of extraction and assay was tested as follows: Five ovariectomized mice were minced and 5 mg. of D.B.E. added and thoroughly mixed in. The mixture was extracted by the method described above and the extract assayed. The recovery

340

was approximately 100%   A similar experiment performed with the liver of mice instead of whole mice, gave the same result   It was difficult to obtain a sufficient quantity of fat and muscle from mice to perform similar control experiments, but they were done on rabbit tissues fat and muscle from rabbit were mixed with definite quantities of D B E extracted and assayed   Again the recovery was complete, within the limit of error   It was also shown that when untreated ovariectomized mice were extracted, the administration of an amount of such an extract corresponding to one ninth of a mouse to each ovariectomized test mouse produced not the slightest oestrogenic effect   In further control experiments extracts from fat and muscle removed from untreated ovariectomized mice were assayed In two experiments muscle extracts produced no response whatever, from which it could be concluded the muscle contained less than 100μgm of the oestrogen per 100 gm of tissue   In one experiment with a fat extract the response was completely negative, showing an oestro-
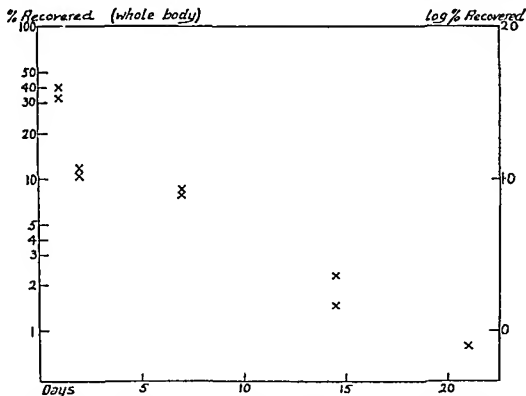
FIG 1 SHOWING THE PERCENTAGE OF A DOSE OF D B E THAT COULD BE RECOVERED FROM THE WHOLE BODY OF MICE AT VARIOUS STAGES AFTER ITS ADMINISTRATION
Each mouse received 2 mg D B E by stomach tube

gen content of less than 300μgm per 100 gm of fat, in another such experiment a very slight response was obtained with the fat extract this was equivalent to an oestrogen content of less than 250 μgm per 100 gm of fat

RESULTS   Ovariectomized mice received 2 mg of D B E in 0 2 c c oil by stomach tube and the whole body was extracted after various intervals   The percentage of the dose recovered is shown in figure 1   It will be seen that more than half of the oestrogen disappeared within the first twenty four hours, but that more than 20% could still be recovered on the third day and between 5 and 10% one week after its administration

In further experiments various organs and tissues were extracted to determine the site of storage

Figure 2 shows the amounts (expressed as mg./100 g. of tissue) recovered from fat and muscle at various stages after the administration of 2 mg. of D.B.E. per mouse (by stomach tube). The figures for the whole body are also inserted, for comparison. It will be seen that the amounts stored in the body fat are larger than in the whole body or muscle; this is especially the case after the first few days following the administration of the substance; the amounts found in the muscle and the whole animal are then very small, while substantial amounts are still present in the fat even two weeks after the administration.
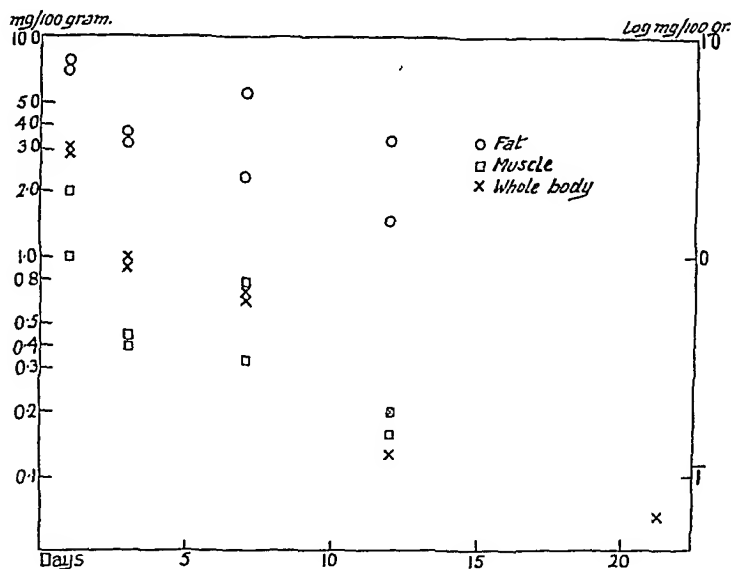
FIG. 2. SHOWING THE AMOUNTS OF D.B.E. (EXPRESSED AS MG. PER 100 GM. OF TISSUE) RECOVERED FROM THE FAT AND MUSCLE (AND FOR COMPARISON, THE WHOLE BODY) OF MICE AT VARIOUS STAGES AFTER ITS ADMINISTRATION
Each mouse received 2 mg. D.B.E. by stomach tube

Evidence was also obtained that the fat is the main storage place in the rabbit. One animal (weighing 1.85 kgm.) received 200 mg. D.B.E. by stomach tube and was killed 24 hours later. The fat contained 6.5 mg./100 gm. while the muscle contained less than 0.2 mg./100 gm. In another rabbit (weighing 2 kgm.) 400 mg. was given by stomach tube and the tissues were removed and extracted five days later. The fat and muscle contents were respectively 2.0 mg. and 0.2 mg./100 gm. of tissue.

In preliminary experiments it was found that very little of the oestrogen was stored in the liver, less than 0.5 mg. per 100 gm. being present 24 hours after the administration. In a more thorough test, five ovariectomized mice received 2 mg. D.B.E. by stomach tube during five consecutive days and the animals

were killed 24 hours after the last dose   The following amounts were found in various tissues

Liver   not more than 0 4 mg /100 gm

Muscle   2 0 mg /100 gm

Fat   15 mg /100 gm

Uterus   The amounts were so small that only a very rough test was possible This suggested that the uterus contained about the same amount as in the muscle i e 2 mg /100 gm

Hence, even with repeated administration, only small amounts were recovered from the liver   This was also found to be true for the rabbit, 400 mg of D B E was given to a 2 0 kgm rabbit by stomach tube, 24 hours later the liver was removed and extracted, it contained about 0 45 mg /100 gm

TABLE 1

Showing the percentage of the amount of D B E administered by stomach tube to groups of ovariectomized mice which was recovered from the excreta

EXCRETA	PERIOD OF COLLECTION	PERCENTAGE OF OESTROGENS ADMINISTERED RECOVERED
	Days	*per cent*
Urine and faeces	1-3	10
	1-4	10
	4-7	<2 5
Faeces	1 3	6
	1-6	8
	4-7	2
Urine	1-3	0 5
	1-6	0 4
	4-7	0 5

In one experiment the oestrogen content of the muscle, skin and bone were determined 24 hours after administration in mice (2 mg per mouse)   The content of muscle and skin were very similar (about 2 0 mg /100 gm) but the bone contained appreciably less (about 0 75 mg /100 gm)   The brains from 10 mice which received 2 0 mg of D B E were removed and extracted 24 hours after the administration   The extract produced not the slightest effect in the test animals, but, because of the small amount of material, it is possible to say only that the brain contained less than 0 5 mg /100 gm of tissue

In a number of experiments assays were performed on extracts of the excreta of mice which had received 2 mg of D B E by stomach tube   The mice were placed in small metabolism cages immediately after the administration   In some experiments the urine and faeces were collected and extracted together, while in others this was done separately   The results are collected in table 1 They show that up to 10% of the dose given is excreted within the first few days but that after this period very small amounts are excreted   Most of the

material excreted appears in the faeces, the urine containing only very small quantities.

Experiments were also performed to determine whether any destruction of the oestrogen could be demonstrated by the liver *in vitro*. Livers from 5 ovariectomized mice were ground and then thoroughly mixed with 5 mg. of D.B.E.; 20 c.c. of saline was added to the mixture which was then incubated at 39°C.; during the period of incubation a slow current of air was passed through the mixture. At the end of incubation the mixture was extracted and assayed by the usual methods. In two separate experiments in which the incubation period was 5 hours the amount of oestrogen recovered was 100%.

DISCUSSION. The results show that the fate of D.B.E. is, in some respects, very different from that of the oestrogens so far studied. Unlike the natural oestrogens, which are rapidly inactivated, chiefly by the liver (Zondek, 1934; Israel et al., 1937; Heller, 1940), D.B.E. is stored in the body tissues to an appreciable extent and is comparatively slowly eliminated. In this respect it differs from stilboestrol which is rapidly eliminated from the body after absorption, an appreciable percentage appearing in the urine (Zondek and Sulman, 1939; Stroud, 1939).

The substance is apparently distributed throughout a number of tissues, though by no means equally. Thus, twenty-four hours after its administration, an oestrogen with the properties of D.B.E. can be extracted from fat, muscle, skin, bone and uterus. The concentration of the substance in the fat is greater than in any of the tissues examined. On the other hand, the liver contains very little and none can be detected in the brain; this suggests that the substance does not pass through the cerebro-spinal barrier. D.B.E. is gradually eliminated from the body and by the end of a fortnight only a small percentage of the dose given can be recovered. The elimination occurs much more rapidly from the rest of the body than from the fat, which contains most of the oestrogen present in the body one week after its administration. The selective retention of D.B.E. by the fat may be due to a greater solubility in adipose than in other tissues of the body.

The loss of D.B.E. from the body is only to a small extent due to excretion in the faeces and urine. Thus the amount present falls by practically 90% during the first three days following the administration, but only about 10% of the dose appears in the excreta, most of it being present in the faeces. It is therefore to be presumed that the substance is metabolised in the body, presumably not by the liver, in view of the results of experiments performed *in vitro*, though these can by no means be regarded as conclusive. Inactive metabolic products may, of course, be excreted in the urine.

In experiments at present in progress (Robson and Adler, 1943) it has been found that D.B.E. does not act directly on the vagina but has to be absorbed into the body before it can produce its effect, *i.e.* it is a pro-oestrogen according to the nomenclature suggested by Emmens (1942). In view of the results described above it seems possible that D.B.E. is stored in the body, and chiefly

in the fat, as such, and that small amounts of it are continually liberated and activated to the actual oestrogen of which the composition is at present unknown

These properties of D B E suggest that it may be suitable for the treatment of conditions in which a prolonged oestrogenic action has to be produced, e g the treatment of menopausal symptoms Clinical investigations with D B E are at present in progress

SUMMARY

D B E was administered to ovariectomized mice by stomach tube Twenty four hours later, less than 50% of the dose could be recovered from the body, but after that the elimination became slower and was almost completed in 3 weeks

The highest concentration of the substance was found in the body fat from which it was more slowly eliminated than from the rest of the body Only 5–10% of the dose given could be recovered from the excreta Most of this was present in the faeces

In vitro experiments suggest that the liver does not play an important part in the inactivation of the substance

The mode of action of D B E is discussed and it is suggested that it may be suitable for the treatment of conditions in which prolonged oestrogenic action is required

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ETHER ANESTHESIA AND THE OUTPUT OF FLUIDS FROM THE RESPIRATORY TRACT

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Inhalation of ether is universally held to augment the flow of saliva and of secretions along the respiratory tract. Thus, for example, Goodman and Gilman (1), discussing the use of atropine in ether preanesthetic medication, state, "The main purpose of the drug is to prevent excessive secretions of the respiratory tract and salivary glands," and, "The inhibition of secretions definitely lessens the incidence of postoperative pulmonary complications."

There are two aspects to statements such as the above. First, there is that relating to the sialogogue action and secondly that related to the increased output of secretions of the respiratory tract, secretions which shall be referred to as R.T.F. or Respiratory Tract Fluid. The sialogogue action is well known and scientifically proven to occur in animals as well as in man. It is generally though not universally believed due (2) to irritation of the sensory nerve endings in the nose, mouth and upper respiratory tract with a consequent reflex flow of saliva since (a) it does not occur if the lining of the upper respiratory tract has been previously locally anesthetized, or (b) if the ether is given intravenously or (c) during "deep" ether anesthesia when the reflexes are inhibited but when pilocarpine is still a sialogogue (3). From studies to be reported below, the anesthesia would seem to have to be very "deep" to inhibit these reflexes bacause even in Plane III of surgical anesthesia a considerable outflow of naso-bucco-pharyngeal secretions occurred.

The reported effect of ether inhalation upon the output of R.T.F. rests upon less secure grounds and mostly by analogy with the sialogogue action. This is because no satisfactory method of collecting, measuring and analysing R.T.F. existed before that published from this laboratory by Perry and Boyd in 1941 (4). Apart from more or less circumstantial evidence, such as that of Lucas and Henderson (5) on the effect of ether upon the bucco-oesophageal mucus of the frog, no difinite information exists upon the action of ether upon the output of R.T.F.

During the course of studies from this laboratory upon the effect of expectorant drugs upon the output and composition of R.T.F. (e.g. (6)), ether has been purposely avoided as an anesthetic agent because of its reputed effect upon the output of R.T.F. It was realized, however, that this reputed effect had never been satisfactorily proven and eventually it was decided to make a study of the question. In the present communication will be described the effect of inhalation of ether upon the rate of production of R.T.F. in rabbits, cats, dogs and guinea pigs. The surprising result was obtained that inhalation of ether, while having a marked sialogogue action, had no or practically no effect upon the volume of R.T.F. in most of the animals and that atropine sulphate in no way

influenced the output of R T F though it dried up the flow of saliva in the usual way. In discussion it will be suggested that these unusual findings may have been due to the probability, as seen from experiments now in progress, that the output of R T F is not markedly affected by drugs acting upon the autonomic system and hence that probably the "tone" of the autonomic nerve endings and associated structures in glands along the respiratory tract is low. Ether, if it acts by reflexes from the upper respiratory tract, could be expected to have little effect upon the output of R T F under these circumstances.

Technique   The method of collecting R T F was that of Perry and Boyd (4) with later improvements described by Boyd Jackson and Ronan (7)   Essentially it consisted in collecting R T F through a T or Y tracheal cannula ligated into a urethanized etherized or decerebrate animal the inhaled air conditioned to temperature and moisture and the whole of the exposed parts insulated   The output of R T F was measured half hourly and expressed as ml per kilo body weight per 24 hours   Freshly redistilled peroxide free ether was used either as the initial anesthetic agent or introduced into the inhaled air of a urethanized or decerebrate animal by blowing air through a vessel of ether and then into the inhaled air in the air conditioning box at any desired rate

TABLE 1

Effect of ether inhalation upon the output of respiratory tract fluid in urethanized animals

SPECIES	URETHANIZED CONTROLS			ETHER INHALATION		
	Number of animals	Mean	S E	Number of animals	Mean	S E
Rabbit	67	2 0	0 05	44	1 9	0 09
Cat	9	2 2	0 2	9	3 0	0 15
Dog	9	0 4	0 08	9	1 3	0 13
Guinea pig	5	3 1	0 56	5	2 9	0 50

The inhalation of ether in urethanized animals   With urethane (ethyl carbamate), the standard anesthetic agent which has been used in all previous studies on R T F in this laboratory, it is difficult to get much below Plane I of surgical anesthesia   Urethanized rabbits, cats, dogs and guinea pigs were arranged for collection of R T F which was measured over a period of three hours or longer and then ether introduced for a further period of three or more hours   The mean outputs of R T F during urethane and the subsequent ether anesthesia were then calculated and these means have been listed in table 1, together with the standard errors of the means (standard deviation divided by the square root of the number of readings)

From the values given in table 1, it is obvious at once that the introduction of ether into the inhaled air had no effect upon the output of R T F in rabbits and guinea pigs   In the cat, the mean output of R T F was increased about one third by ether inhalation and the mean difference of 0 8 ml /kilo/24 hrs had a standard deviation of 0 25 giving a P value less than 0 05 and hence statistically significant   From certain other considerations, however, it seems doubtful if ether really does increase the output of R T F in spite of the apparent statistical significance of the experiment just described   For example four of the nine cats

used in this experiment survived until the second day or even longer and beginning the morning of the second day they had been without ether for at least 15 hours. These 4 cats were given no ether the second day in order to find out if the rate of output of R.T.F. would fall after ether anesthesia, the cats still being anesthetized with urethane. Taking these 4 cats only, their mean output of R.T.F. during the inhalation of ether had been 3.3 ml./kilo/24 hrs. the previous afternoon and throughout the second day the mean rate was found to be also 3.3 ml./kilo/24 hrs. During the second day, there was no smell of ether to the expired air although it is quite possible that small quantities may have been present as ether elimination by way of the lungs continues for some time after ether inhalation. The failure of the output of R.T.F. to decline after inhalation of ether had been discontinued for some time did, however, throw some doubt on the validity of the previous conclusion that ether inhalation increased the output of R.T.F. in cats. This doubt was crystallized into almost certain conviction when it was found, as will be described in the next section, that the inhalation of ether by decerebrate cats did not augment the output of R.T.F. The conclusion was reached, therefore, that ether inhalation had little if any effect upon the output of R.T.F. in cats and certainly none in rabbits and guinea pigs.

On the other hand, in the dog ether did increase the output of R.T.F. some threefold or so. As seen in table 1, the mean difference between the output of R.T.F. in urethanized and urethanized, etherized dogs was 0.9 ml./kilo/24 hrs., the increase following the inhalation of ether. This mean difference was calculated to have a standard deviation of 0.15 ,which of course is highly significant statistically, and as an increase was found in all but one of the 9 dogs used, it may be concluded that ether inhalation does augment the output of R.T.F. in dogs. Furthermore, 4 of the 9 dogs lived overnight, during the second day they were given no ether and the mean output of R.T.F. definitely fell; the rate of output under ether of these particular 4 dogs had been 1.82 and during the second day the mean rate fell to 0.99 ml./kilo/24 hrs., the mean difference of 0.83 having a standard deviation of 0.22. These data indicate pretty clearly that ether inhalation increases the output of R.T.F. in dogs and that after cessation of ether inhalation, the output of R.T.F. returns toward the original.

There is an objection which might be raised to the above methods of calculating the effect of ether—or lack of effect—upon the output of R.T.F., namely that mean rates have been compared over several hours before and after ether, rather than hourly changes in the rate of output of R.T.F. By the latter method, it might be offered in criticism, a effect of ether might be demonstrated, an effect which could be obscured by the computation of means. Hence the hourly rates of output of R.T.F. have been averaged and plotted in figure 1 for all of the cats, dogs and guinea pigs and for a representative group of rabbits. It is obvious from figure 1, that the conclusions previously reached from a calculation of means have been in no way affected by this added method of expressing the results.

A second possible criticism to the conclusion that ether did not augment the output of R.T.F. in rabbits, cats and guinea pigs might be that the combination

of urethane and ether depressed reflexes to the point where the irritant ether fumes could not initiate a reflex stimulation of the output of R.T.F. While the degree of anesthesia varied somewhat, on the average most of the animals were in Plane II of surgical anesthesia   It will be shown later that the output of R.T.F. is not materially influenced by changing the degree of anesthesia amongst Planes I, II and III and that the output of salivary secretions continues at an augmented
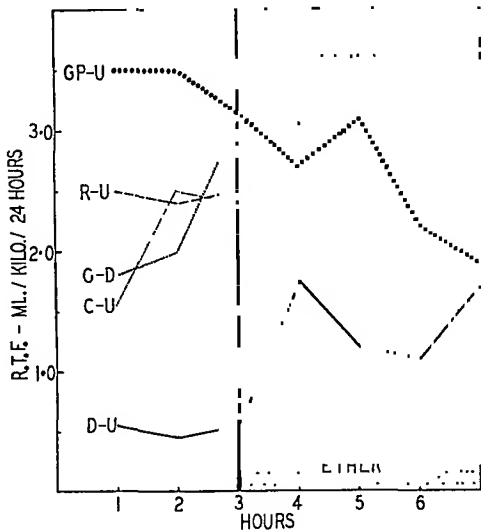
FIG. 1. EFFECT OF ETHER INHALATION UPON THE OUTPUT OF R.T.F.

GP-U: Guinea pigs given urethane before ether, R-U: rabbits given urethane before ether; C-D: decerebrate cats with no anesthetic before ether, C-U: cats given urethane before ether; D-U: dogs given urethane before ether.

rate in all three planes.   Hence it would not seem likely that the degree of anesthesia used was sufficient to decrease the output of R.T.F. by inhibiting possible reflexes to the respiratory tract mucosa and its glands.

The inhalation of ether by decerebrate cats. Although it seemed unlikely that urethane anesthesia had affected the output of R.T.F. in the animals described in the previous section, nevertheless it was deemed advisable to investigate the output of R.T.F. in decerebrate cats without anesthesia and later after given

either urethane or ether anesthesia. The mean rates of output of R.T.F. of non-anesthetized, decerebrate cats collected over a period of 3 hours or more and the subsequent means when given either urethane at a dose of 1 gm. per kilo interstitially or plane 2 of ether anesthesia by inhalation into the tracheal cannula, are shown in table 2 and the mean hour by hour effect is charted in figure 1.

It may be seen that neither urethane nor ether significantly altered the rate of output of R.T.F. from that in the non-anesthetized, decerebrate cat. The conclusion from these experiments is that ether has no effect upon the rate of output of R.T.F. in cats. It was further gratifying to find that the output of R.T.F. under urethane anesthesia, which has been used as the "control" rate in previous studies from this laboratory, was the same as in decerebrate cats. While urethane is not a powerful anesthetic agent, it does dilate the bronchi a bit and these considerations made it desirable to compare the output of R.T.F. in urethanized and in non-anesthetized, decerebrate cats.

The output of R.T.F. in different planes of ether anesthesia. Reference has been occasionally made in the previous sections of this communication to the planes of ether anesthesia and in this section will be described experiments which were

TABLE 2

Effect of urethane and ether anesthesia upon the output of respiratory tract fluid in decerebrate cats

NUMBER OF ANIMALS	NO ANESTHESIA	ANESTHESIA	
	Mean R T.F.	Anesthetic agent	Mean R.T.F.
5	2.1	Ether	2.3
11	1.8	Urethane	1.8

designed to investigate if the output of R.T.F. was affected by the plane of ether anesthesia. As pointed out by Sollmann (2), the sialogogue action of ether varies with the degree of ether anesthesia. Hence it was also possible that the output of R.T.F. might be affected by the degree of anesthesia. The output of R.T.F. was studied in rabbits, cats and dogs under Planes I, II and III of ether anesthesia, the planes being adjudicated after Guedel's classification of signs (8).

The animals were anesthetized initially with ether (not urethane) and prepared for collection of R.T.F. They were kept in one plane for 2 or more hours and then shifted to another, varying the sequence so as to produce a random sampling of planes. The mean output of R.T.F. in the different planes was then calculated and the data are given in table 3. There were no marked differences in the output of R.T.F. in the different planes of ether anesthesia. The mean output was greatest in Plane I with cats and dogs and in Plane III with rabbits but the differences between the lowest rate, highest rate and middle rate were not great.

It may be of interest at this point to note the effect of the different planes of ether anesthesia upon the output of saliva. No accurate record was kept of the outflow of saliva but in some 15 rabbits an estimate was made of the volume of fluid, probably mostly saliva, which dripped from the nose and mouth by col-

lecting it in a beaker and measuring its volume   In the urethanized or decerebrate animals, no or very little fluid dripped from the nose and mouth   When ether was used as an initial anesthetic agent, there was a considerable output of this fluid which, for brevity's sake, will be referred to as "saliva"   The flow of "saliva" continued throughout the period of ether inhalation, irrespective of the plane of anesthesia   In the 15 rabbits where a rough estimate was made, the average output of "saliva" was almost identical in Planes I, II and III at about 25 ml /kilo/24 hrs   The true volume was undoubtedly higher because some, possibly considerable, of the fluid evaporated from the nose and mouth and while lying in the open beaker beneath   The chief point brought out by this rough experiment was that the reflex flow of "saliva" from the irritating effect of ether was not depressed in Plane III of anesthesia and Plane III was the deepest plane used in all of these studies of R T F production

The effect of ether upon the ciliary drainage of R T F   It is well known to every pharmacologist that if sufficient ether be added to solution bathing cilia, ciliary function is depressed   The mucosa lining the mammalian respiratory tract is ciliated from the terminal bronchioles to the nose   This ciliated carpet, together

TABLE 3

Comparison of the output of respiratory tract fluid in Guedel's planes of ether anesthesia

SPECIES	RABBIT	CAT	DOG
Number of animals	25	10	9
Plane I R T F	1 4	3 7	1 7
Plane II R T F	1 9	2 9	1 2
Plane III R T F	2 4	3 0	1 2

with certain other mechanisms which have been discussed by Boyd and Ronan (9), is responsible for the excretion of R T F   If ether depressed the function of these cilia, then it is possible that ether might have caused an increased production of R T F in the sections of this report described above but the augmented output could not be all eliminated and remained lying in the respiratory passages   If so, then by postural drainage of the lung in the Trendelenburg position with the axis of the body held head downward, the accumulated R T F should be readily drained away and the output of R T F should be thereby increased

To investigate this possibility, rabbits, cats and dogs were anesthetized with ether and arranged for collection of R T F upon especially made tables, described by Boyd, Jackson and Ronan (7), by which the animals could be arranged for postural pulmonary drainage in a modified Trendelenburg position   R T F was collected (a) with the animals lying prone upon their bellies, (b) with the animals arranged for pulmonary drainage with the axis of the body held head downward at an angle of 30° and (c) 50° with the horizontal over a period of 1 day

The mean output of R T F with the lungs not posturally drained and with drainage at the two different angles, is listed in table 4   It is quite evident that

postural pulmonary dráinage did not increase the output of R.T.F. during ether anesthesia. These results prove definitely that ether anesthesia does not materially alter the rate of production or output of R.T.F. in rabbits and cats and that ciliary drainage of R.T.F. is not impaired in ether anesthesia of rabbits, cats and dogs.

The effect of atropine sulphate upon the output of R.T.F. The time-honoured use of atropine sulphate, "to prevent excessive secretions of the respiratory tract," (1) naturally suggested a trial of its effect upon the output of R.T.F. as herein measured. Given subcutaneously in doses ranging up to the lethal dose to rabbits, cats and dogs under urethane or ether anesthesia, it had no consistent effect whatsoever upon the output of R.T.F. The results, showing the mean output of R.T.F. during 3 or more hours before and for a corresponding period

TABLE 4

Effect of different angles of postural drainage of the respiratory tract upon the output of respiratory tract fluid during ether anesthesia

SPECIES	RABBIT	CAT	DOG
Number of animals.....................	30	29	21
R.T.F. at angle 0°........................	2.4	3.3	1.9
R.T.F. at angle 30°.........	2.4	2.8	1.4
R.T.F. at angle 50°.....................	2.6	2.6	1.3

TABLE 5

Effect of atropine sulphate upon the output of respiratory tract fluid

SPECIES	RABBIT		CAT	DOG
Anesthetic agent.....................	Ether	Urethane	Ether	Ether
Number of animals..................	17	9	11	4
Pre-atropine R.T.F..................	2.3	1.5	3.3	1.0
Post-atropine R.T.F................	2.3	1.9	2.8	0.8

after atropine, are given in table 5. The doses of atropine sulphate used were from 50 to 500 mgm. per kilo for rabbits, 1 to 20 mgm. per kilo for cats and 1 to 4 mgm. per kilo for dogs. In all these experiments, atropine markedly reduced the flow of saliva which with ether was greatly increased but it had little or no "drying up" effect upon the secretion of R.T.F.

The absence of any inhibitory effect of atropine upon the output of R.T.F. was an unexpected result, especially in cats and dogs. Rabbits tolerate large doses of belladonna alkaloids, their "cholinergic tonus" is in general low and they have few acinar glands in the respiratory tract, so that the lack of any action from atropine was not entirely unexpected. The mucus-secreting goblet cells are sparingly, if at all, innervated by autonomic nerves (10) and do not respond to cholinergic drugs (11). On the other hand, cats have plenty of acinar glands in the respiratory tract, glands which are freely innervated and which have been

shown to secrete when cholinergic drugs are given (11)   Dogs likewise are well
supplied with acinar glands in the respiratory tract   On the other hand, Boyd
and Jackson (12) find that the cholinergic drugs, Carbachol B P ('Doryl'),
pilocarpine nitrate and physostigmine salicylate have little effect upon the output
of R T F in urethanized cats except in sub lethal doses   Since adrenergic drugs
have little effect upon the output of R T F (7), it would appear that the glands
of the respiratory tract are not particularly sensitive to adrenergic or cholinergic
stimulation   The advantage of atropine in ether pre anesthetic medication is
obviously prevention of the sialogogue action and not a diminution of secretion of
R T F , that is at least in rabbits, cats and dogs   Whether the same statement
applies to man cannot be said from the evidence available and especially in view
of the species variation which, from the present studies, would appear to be a
characteristic of R T F production

SUMMARY

Anesthetized or decerebrate cats, rabbits, dogs and guinea pigs were arranged
for collection of R T F (Respiratory Tract Fluid) through a tracheal cannula
and with conditioned inhaled air

The inhalation of ether had no effect upon the output of R T F in urethanized
rabbits, cats and guinea pigs but did increase the output some threefold in dogs

The output of R T F in rabbits, cats and dogs was not different in Planes I,
II and III of ether anesthesia

The output of R T F in decerebrate cats was similar to that in urethanized
cats and not affected by the inhalation of ether

There was no evidence that the inhalation of ether damaged the cilia lining the
respiratory airway and which drain R T F because the output of R T F under
ether anesthesia was not increased by postural pulmonary drainage

Finally, atropine sulphate did not affect the output of R T F in urethanized
nor in etherized rabbits, cats and dogs

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STUDIES ON SULFAPYRAZINE

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In 1941 Ellingson (1) synthesized a sulfonamide (2-sulfanilamidopyrazine) which he named sulfapyrazine. Studies by Schmidt et al. (2, 3) showed that sulfapyrazine compared favorably with sulfadiazine, sulfapyridine and sulfathiazole in experimental pneumococcal and streptococcal infections in mice. Their results also indicated that sulfapyrazine possessed greater activity *in vitro* against pneumococci than sulfadiazine. Preliminary clinical studies (4) suggest that patients tolerate sulfapyrazine about as well as sulfadiazine and better than sulfapyridine or sulfathiazole. Little has been reported, however, on the efficacy of this compound against other bacterial infection or the possible toxic effects following prolonged administration of the drug. The results presented in this report are concerned with these aspects.

GENERAL COURSE OF THE INVESTIGATION. The experiments which are included in this report may be divided into two main groups. In group I the toxicity of sulfapyrazine was compared with that of sulfadiazine using the effects of the acute, cumulative and chronic administration as a basis for comparison. In group II, sulfapyrazine, sulfadiazine, sulfapyridine and sulfathiazole were compared for their relative effectiveness in experimental streptococcal, pneumococcal and staphylococcal infections in mice.

MATERIALS. Male Swiss mice (CF₁) and rats (Carworth) were used throughout the course of this investigation. The animals were maintained on an adequate stock diet with sufficient water available at all times. In the acute toxicity and in the efficacy experiments the drugs were ground in a mortar and suspended in 10% gum acacia. For the chronic toxicity studies the drugs were incorporated in the stock diet and fed to animals ad libitum. Samples of the drug diet mixture were taken at random and analysed for drug content in order to be certain that the mixture was homogeneous.

Strains of *Streptococcus hemolyticus* (1685), *Diplococcus pneumoniae* (type I) and *Staphylococcus aureus* (Smith) were used to produce experimental infection. The organisms were grown in brain heart infusion media supplemented with 10 per cent defibrinated rabbit blood. The degree of virulence of all strains was such that 0.5 cc. of a 10^{-4} culture dilution provided an infection ranging from 10,000 to 20,000 lethal doses as determined by titration in mice. Virulence was maintained by mouse passage twice each week. The drug concentration in the blood of test animals was determined using the method of Bratton and Marshall (5). In the efficacy experiments dealing with mice, blood level determinations were usually performed on a group of uninfected mice treated with the drug in the same manner as the infected animals.

RESULTS. *Acute and cumulative toxicity.* Acute oral toxicity experiments

indicate that single doses of sulfapyrazine or sulfadiazine are not lethal for mice, rats or dogs even in quantities as large as 10 grams per kgm (table 1) Dogs fed either drug by stomach tube showed no tendency to vomit such as reported for sulfapyridine (6) When doses of 0 5, 1 0, 2 0 or 4 0 grams per kilogram of either drug were administered daily over a 10 day period to mice, no outward

TABLE 1

Acute oral toxicity of sulfapyrazine and sulfadiazine in mice rats and dogs

| | DOSE | NO OF ANIMALS PER DOSE | PERCENTAGE DEAD | |
			Sulfapyrazine	Sulfad as ne
	gm /kgm			
Mice	1 0	50	0	0
	5 0	50	0	0
	7 5	50	0	0
	10 0	50	0	0
Rats	1 0	25	0	0
	5 0	25	0	0
	7 5	25	0	0
	10 0	25	0	0
Dogs	1 0	7	0	0
	5 0	7	0	0

TABLE 2

Cumulative toxicity of sulfapyrazine and sulfadiazine in mice

| | DOSE* | NO OF MICE PER DOSE | NO DEAD IN DAYS | | | | | | |
			1	2	3	4	5	10	15
	gm /kgm								
Sulfapyrazine	0 5	50	0	0	0	1	0	0	0
	1 0	50	0	0	0	0	0	1	0
	2 0	50	0	0	0	0	0	0	0
	4 0	50	0	0	0	0	1	0	0
Sulfadiazine	0 5	50	0	0	0	0	0	1	0
	1 0	50	0	0	0	0	0	1	0
	2 0	50	0	0	0	0	0	1	0
	4 0	50	0	0	0	0	0	0	0

* Fed daily for 10 days

signs of toxicity were observed (table 2) Animals sacrificed at the end of the feeding period showed no abnormalities upon gross examination The maximum drug concentration in the blood in the 10 day feeding experiment was 38 0 mgm per cent for sulfapyrazine and 55 4 per cent for sulfadiazine Therefore, the lack of oral toxicity of sulfapyrazine or sulfadiazine appears to be due to the limited amount of drug absorbed from the intestinal tract Visible amounts of the drugs were observed in the feces of the foregoing animals

Toxicity of sulfapyrazine-sodium. This was determined in mice by administering a 10 per cent solution intravenously in doses ranging from 0.25 to 1.0 grams per kgm. Observations were made daily over a seven-day period. As shown in table 3, sulfapyrazine-sodium is definitely toxic under these conditions. This toxicity, however, is not greater than that reported for sulfadiazine-sodium.

Chronic toxicity in mice, rats and dogs. Sulfapyrazine and sulfadiazine were incorporated in powdered stock diet (S₁)[1] in concentrations of 0 5 and 1 0 per cent and fed to mice and rats over a period of five months. An equal number of animals fed this stock diet served as controls. Throughout the course of the experiment, food and water consumption and the weight of the rats were recorded.

Both drugs were found to influence the normal development of rats in the 0.5 and 1 per cent drug concentration as judged by the weight curves At first

TABLE 3

Acute intravenous toxicity of sulfapyrazine-sodium in mice

DOSE	NO OF MICE PER DOSE	NO DEAD IN DAYS							TOTAL NO DEAD
		1	2	3	4	5	7	10	
gm /kgm									
0.25	10	0	0	0	0	0	0	0	0
0.50	10	0	0	0	0	0	0	0	0
0 75	10	0	0	1	1	0	0	0	2
1.0	10	5	3	2	0	0	0	0	10
2 0	10	9	1	0	0	0	0	0	10
2 5	10	8	1	1	0	0	0	0	10
3 0	10	9	1	0	0	0	0	0	10
3.5	10	9	1	0	0	0	0	0	10
4.0	10	10	0	0	0	0	0	0	10

this effect appeared to be somewhat greater with sulfadiazine, but eventually there was essentially no difference between the weight of the rats fed 1 per cent sulfadiazine or sulfapyrazine. This retardation of the normal weight gain appeared to be closely associated with a decrease in food consumption. Within two to three weeks the rats on the 1 per cent drug diet mixture of sulfapyrazine or sulfadiazine were only eating about ⅔ to ¾ the amount of food consumed by the control rats. This difference became more striking as the experiment progressed and suggests definite abnormalities in the rats receiving the drugs.

[1] S₁ Diet:

Whole milk powder (Klim)	210 grams
Yellow corn meal	330 grams
Whole wheat, ground	330 grams
Linseed oil meal, old process	70 grams
Alfalfa leaf meal	20 grams
Sodium chloride	5 grams
Calcium carbonate	5 grams
Cod liver oil	100 cc.

Deaths began to occur after the nineteenth day and upon autopsy rats dying from both drugs showed considerable urolith formation which frequently produced a marked degree of hydronephrosis As may be seen in table 4, an equal number of deaths occurred in the groups receiving 0 5 or 1 per cent sulfapyrazine and 1 per cent sulfadiazine No deaths occurred in rats receiving 0 5 per cent sulfadiazine Death appeared to be the result of the hydronephrosis caused by the urolith formation The blood concentration studies indicate that rats on 0 5 or 1 per cent sulfapyrazine had approximately the same level This would explain the similarity in the number of deaths in both groups of rats It is to be noted that the blood level concentrations were influenced somewhat by the decreased food consumption of rats on the 1 per cent drug diet mixture This might account in part for the similarity in the blood levels and toxicity found in rats receiving 0 5 or 1 per cent sulfapyrazine That these results cannot be explained entirely by the effect of food consumption, however, is shown by the fact that the rats receiving the diet containing 0 5 and 1 per cent sulfadiazine

TABLE 4

Chronic toxicity of sulfapyrazine and sulfadiazine in rats

NO OF RATS	DRUG*	CONCENTRATION OF DRUG IN DIET	NUMBER DEAD IN 7 MONTHS	MEAN BLOOD CONCENTRATION	AVERAGE WEIGHT OF SURVIVING ANIMALS
		per cent		*mgm per cent*	
15	Sulfapyrazine	0 5	8	30 0	260
15	Sulfapyrazine	1 0	8	34 8	158
15	Sulfadiazine	0 5	0	28 7	392
15	Sulfadiazine	1 0	9	47 5	180
15	Control	0	0	0	452

* Drugs fed daily for seven months

had a mean blood concentration of 27 7 and 47 5 mgm per cent It will be recalled that sulfadiazine had the same effect upon the food consumption as sulfapyrazine The results show however that more rats died on the 0 5 per cent sulfapyrazine drug diet mixture than on 0 5 per cent sulfadiazine mixture

The findings in the chronic toxicity studies in mice show that both drugs produce kidney concretions in mice It will be recalled, that concretions do not occur in mice following the administration of other sulfonamides such as sulfapyridine or sulfathiazole (6)

When dogs were fed 0 5 and 1 gm per kgm daily over a three month period, uroliths were found in the bladder and kidneys of all animals Since dogs do not acetylate the sulfonamides (7), this indicates that free sulfadiazine or sulfapyrazine can cause kidney concretions

Influence of alkalization of the urine on urolithiasis Clinical experience has shown that the incidence of urolithiasis following sulfadiazine administration is greatly reduced by alkalization of the urine (9) Examination of the solubilities of sulfapyrazine and acetyl sulfapyrazine at various pH levels indicates that the

solubility of both drugs is increased considerably by increasing the pH of the solvent from 7.0 to 7.4 at 37°C. (fig. 1).[2] Similar findings have been reported for sulfadiazine (8).   The explanation of this phenomenon is that these drugs and their acetyl derivatives are weak acids which ionize and form soluble salts in an alkaline medium (9).   One might expect therefore that alkalization of the urine would also have a favorable influence on the incidence of urolithiasis in animals fed sulfapyrazine.   To determine this, two groups of ten rats each were set out on a stock diet containing 1 per cent sulfapyrazine.   One per cent so-
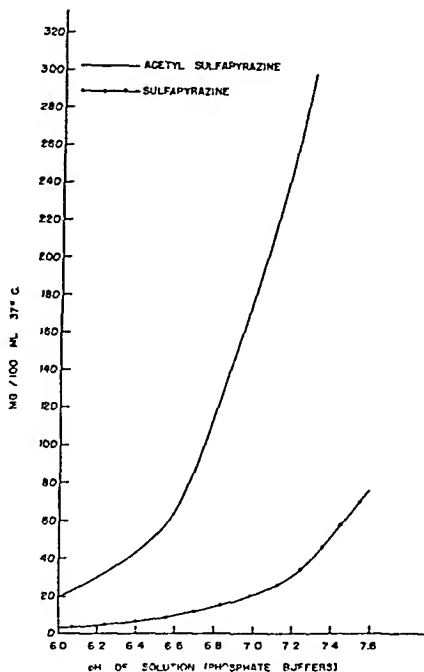
FIG. 1. SOLUBILITY OF SULFAPYRAZINE AND ACETYL SULFAPYRAZINE IN PHOSPHATE BUFFERS

dium bicarbonate was added to the diet of one of the two groups in order to maintain the urine above pH 7.0. For comparison similar experiments were performed with sulfadiazine. No evidence of urolithiasis was found in rats fed the sodium bicarbonate whereas all of the animals not receiving sodium bicarbonate developed concretions and kidney complications. This suggests that alkalization of the urine should be of definite value in reducing or eliminating urolithiasis in the clinical use of sulfapyrazine.

Pathologic and hematologic findings. The rats receiving sulfadiazine or sulfapyrazine revealed the same qualitative pathologic changes, namely, a) concre-

tions in the kidney pelvis ureter and bladder with secondary hydronephrosis, pyonephrosis or pyelonephritis b) markedly hyperplastic thyroid, c) atrophy of the spleen, and d) slight to moderate parenchymatous degeneration of the liver   The difference in the pathologic effects of the two drugs was quantitative On the same dose level rats receiving sulfapyrazine developed the lesions some what earlier and died sooner than those receiving sulfadiazine

The blood findings in rats receiving 1 per cent sulfadiazine or 1 per cent sulfapyrazine in the diet and fed ad lib for six months were normal

All the dogs receiving 0 5 gm or 1 0 gm per kgm sulfapyrazine daily for three months revealed greenish yellow concretions in the kidney pelvis and bladder   The ureters were normal   Microscopic sections revealed a mixture of crystals, fibrin red cells and desquamated epithelial cells in the pelvis   Eosinophilic coagula and occasional red cells were present in Bowman's space the convoluted tubules, and the papillary ducts   A mild chronic pyelonephritis was present   Occasionally severe congestion and hemorrhage of the renal papillae was observed

The livers of the dogs receiving 1 gm per kgm revealed moderately severe venous congestion   The liver cord cells were markedly hypertrophied   The nuclei were normal and an occasional binucleated cell was present   The cytoplasm was increased thinned and granular and occasional large vacuoles containing sudanophilic material were present

The spleens were all slightly smaller than normal   Microscopically the spleen of only one dog receiving 1 g per kgm showed changes   The travecular veins contained early thrombi and scattered throughout the red pulp were small foci of nucleated red cells

The thyroids were all slightly enlarged and congested   The striking change microscopically was the almost complete absence of colloid (fig 2)   The changes were diffuse   There was no increase in connective tissue   The follicles were increased in size   The epithelial cells were tall columnar, and papillation was quite evident   Many desquamated epithelial cells filled the follicles   No mitotic figures were observed   These changes were more marked in those dogs receiving the higher doses

Other tissues showed no abnormal changes except for the presence of a slight bilateral pleural effusion and ascites in two of the four dogs

The blood findings in these dogs were normal

The pathologic findings on sulfadiazine have been reported by others (10) and therefore are not included in this report

Blood levels   In order to evaluate and discuss the results obtained in the efficacy experiment data on the blood concentration of each drug during the period of treatment must be available   For this reason the following experiments were undertaken   Groups of normal mice were fed 5 and 10 mgm doses of each drug depending upon the treatment schedule used during the efficacy experiments   At hourly intervals the blood of five mice was pooled and analyzed for the free drug according to the method of Bratton and Marshall (5) The results of these experiments are given in figure 3

All four drugs reach a maximum blood concentration within 30 to 60 minutes. This concentration is maintained more constant by sulfapyrazine than by the

FIG. 2. HYPERPLASIA OF THE THYROID IN DOGS FOLLOWING SULFAPYRAZINE ADMINISTRATION
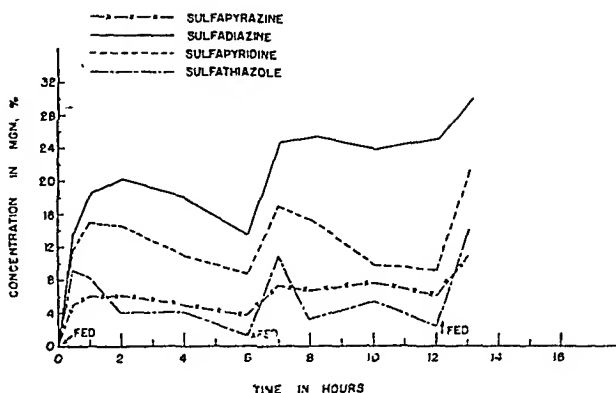
FIG. 3. BLOOD CONCENTRATION OF SULFAPYRAZINE, SULFADIAZINE, SULFAPYRIDINE AND SULFATHIAZOLE IN MICE

other drugs. Doses of 5, 10 or 20 mgs. of sulfapyrazine produce the same blood concentration in mice. Sulfadiazine produces blood concentrations which are considerably higher than any other drug but this is not maintained as well as

sulfapyrazine Sulfapyridine concentrations were greater than sulfapyrazine, but showed considerable fluctuation between the maximum and minimum concentration Sulfathiazole concentrations were somewhat greater than sulfapyrazine levels during a short interval following the treatment but this level declined and was below that of sulfapyrazine most of the time These results are in close agreement to those reported by other investigators (2)

THERAPEUTIC EFFICACY *Methods* The *in vivo* activity of sulfapyrazine was compared with that of sulfadiazine sulfapyridine and sulfathiazole Strains of *Streptococcus hemolyticus* (#1685), *Diplococcus pneumoniae* (Type I #37), and *Staphylococcus aureus* (Smith) were used to produce experimental infection The cultures were grown for six hours at 37°C in brain heart infusion media supplemented with 10% defibrinated rabbit blood

TABLE 5

Comparative activity of the sulfonamides in streptococcal, pneumococcal and staphylococcal infections in mice

Infection 0 5 cc of a 10^{-4}, 10^{-8} or 10^{-8} culture dilution I P
Age of culture 6 hours
Treatment Fed by mouth every 6 hours for 5 days
Dose 5 mgs per mouse

NO OF LETHAL DOSES INJECTED	CULTURE	PERCENTAGE SURVIVAL AFTER 10 DAYS				
		SPA *	SD	SP	ST	Control
50 to 100	St hemolyticus (#1685)	88	90	76	80	0
	D pneumoniae (#37)	80	82	70	72	0
	Staph aureus (Smith)	85	88	80	82	0
800 to 1000	St hemolyticus (#1685)	80	85	50	48	0
	D pneumoniae (#37)	80	84	50	52	0
	Staph aureus (Smith)	85	86	70	72	0
10 000 to 20 000	St hemolyticus (#1685)	60	70	42	36	0
	D pneumoniae (#37)	52	60	18	20	0
	Staph aureus (Smith)	70	74	60	64	0

* SPA , sulfapyrazine SD sulfadiazine, SP , sulfapyridine, ST , sulfathiazole

Infection was produced by the intraperitoneal injection of 0 5 cc of a 10^{-4}, 10^{-6} or 10^{-8} culture dilution This quantity of culture was equal to 10 000 1 000 and 100 lethal doses respectively as determined by titration tests in mice In order to produce a fatal infection with *Staphylococcus aureus* (Smith) the organisms were injected in 4 per cent gastric mucin The infected animals were treated by stomach tube immediately after the bacterial infection and thereafter at six hour intervals day and night for five days

RESULTS *Comparative activity of sulfapyrazine, sulfadiazine, sulfapyridine and sulfathiazole when administered in equal doses* When the experimental infection was not severe (100 lethal doses) there was essentially no difference in the activity of the four drugs All compounds afforded excellent protection to mice in streptococcal, pneumococcal and staphylococcal infections (table 5) When the infecting dose was increased to 1,000 lethal doses, sulfapyrazine and sulfadiazine were distinctly more efficacious than sulfapyridine or sulfathiazole

Under these conditions, sulfapyrazine and sulfadiazine showed essentially the same activity. However, when severe experimental conditions were employed, such as those obtained by injecting 10,000–20,000 lethal doses of organisms, sulfadiazine appeared somewhat more active than sulfapyrazine. From the blood concentration studies, however, it is apparent that the blood levels obtained with sulfadiazine are considerably greater than those of sulfapyrazine. Sulfadiazine might therefore be expected to show a greater activity when the experimental infection is of a severe nature.

Comparative effectiveness of sulfapyrazine, sulfadiazine, sulfapyridine and sulfathiazole at equal blood concentrations. In order to compare the efficacy of these drugs under identical conditions, the dosage was adjusted and administered in such a manner that the average blood concentrations maintained were similar. For this purpose doses of each drug were selected which maintained a blood concentration between 4 and 6 mgs. per cent. Thus quantities of 1.0 mg. of

TABLE 6

Comparative activity of the sulfonamides in mice at equal blood concentrations

Infection: 0.5 cc. of a 10^{-5} culture dilution I.P.

Age of Culture: 6 hours.

Treatment: Fed by mouth every 6 hours for 5 days.

Dose: Adjusted to maintain blood concentrations between 4 and 6 mg. %.

NO. OF LETHAL DOSES INJECTED	CULTURE	PERCENTAGE SURVIVAL AFTER 10 DAYS				
		SPA*.	SD.	SP.	ST.	Control
10,000 to 20,000	St. hemolyticus (#1685)	60	58	50	52	0
	D. pneumoniae (#37)	50	48	44	46	0
	Staph. aureus (Smith)	68	70	65	68	0

* SPA., sulfapyrazine; SD., sulfadiazine; SP., sulfapyridine; ST., sulfathiazole.

sulfadiazine, 5 mgs. of sulfapyrazine, 7 mgs. of sulfathiazole and 6 mgs. of sulfapyridine were given every six hours to mice infected with 1,000 lethal doses of *Streptococcus hemolyticus* #1685. A summary of these results shows that the activity of all four drugs was essentially equal under these conditions (table 6).

DISCUSSION. The results of the acute and chronic toxicity show that sulfapyrazine like sulfadiazine produces kidney concretions in mice, rats and dogs following prolonged administration. Since dogs do not detoxify the sulfonamides by acetylation (7) it is apparent that the free form of these drugs may also cause kidney concretions. This does not occur with sulfapyridine (6). In rats, the incidence of concretion formation appears to be greater with sulfapyrazine than with sulfadiazine. However, since with both drugs the formation of kidney concretion can be prevented by alkalization of the urine, the factor of urolith formation is greatly minimized.

In general, the same pathological changes were produced by sulfapyrazine and sulfadiazine in rats and dogs.

Sulfapyrazine, like sulfadiazine proved to be a potent drug against experi-

mental streptococcal, pneumococcal and staphylococcal infections in mice   In mild or moderately severe infections, sulfapyrazine and sulfadiazine were more active than sulfathazole or sulfapyridine   As pointed out recently by Schmidt and Sesler (3) the superiority of sulfapyrazine and sulfadiazine over other sulfonamides appears to be due to the slow rate of absorption and excretion of these drugs making it possible to maintain effective blood concentrations be tween doses   Thus when the dosage was adjusted to afford equal blood concentrations, all drugs showed essentially the same activity   The results of Marshall et al (11) and Schmidt et al (2) indicate that sulfapyrazine is even more effective than sulfadiazine in experimental pneumococcus infections in mice when compared on the basis of equal blood concentrations

The findings reported in this paper suggest that sulfapyrazine, like sulfadiazine, would make a useful addition to the field of bacterial chemotherapy, providing clinical findings bear out the experimental studies

SUMMARY

1 Rats receiving sulfapyrazine or sulfadiazine revealed the same qualitative pathological changes following prolonged administration

2 The incidence of urolith formation following the administration of sulfadiazine and sulfapyrazine can be greatly reduced or completely eliminated by alkalization of the urine

3 Sulfapyrazine and sulfadiazine appear to be equally effective against experimental pneumococcal, streptococcal, and staphylococcal infections when compared on the basis of equal blood concentrations

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STUDIES ON PHYSOSTIGMINE AND RELATED SUBSTANCES[1]

IV. CHEMICAL STUDIES ON PHYSOSTIGMINE BREAKDOWN PRODUCTS AND RELATED EPINEPHRINE DERIVATIVES[2]

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Interest in the colored decomposition products of physostigmine began as early as did the attempts to prepare a pure alkaloid from the calabar bean. Thus, in the work of Jobst and Hesse (1) and Hesse (2) in which alkali was used to precipitate the alkaloid or to aid in the extraction with ether, the authors noted the red color which rapidly appeared in alkaline solutions of physostigmine. Their observation that the color formation was slow in sodium bicarbonte solution, much more rapid in sodium carbonate solution, and immediate in ammonium hydroxide solution may be interpreted to mean that the hydroxyl ion concentration controls the rate of formation of this red color [see paper No. II (3) of this series for a detailed study of the rate of destruction and its dependence upon pH]. Under certain conditions blue coloration in the physostigmine preparations was also detected by these authors.

Much information about these colored products had been brought to light even before Polonovsky (4) was in a position to propose the possible structures of physostigmine and one of the postulated structures was proven to be correct by its synthesis (5, 6). Thus, the red substance which causes the characteristic pink color of decomposed physostigmine solutions was isolated and named rubreserine by Duquesnel (7). A blue substance was obtained by Petit (8) when physostigmine was evaporated with concentrated ammonia on the steam bath and was called eserine blue. The product obtained by Ferreira da Silva (9) by evaporating physostigmine to dryness with nitric acid was called chloreserine because of its green color.

The mechanism of the decomposition of the drug was first studied by Ehrenberg (10) and later by Salway (11). The latter gave methods for preparing the products in pure form. The first step in the breakdown was shown to involve the loss of methylamine and carbon dioxide, leaving a product which Salway found to be a phenolic compound, eseroline. This phenolic compound was then oxidized to a red compound, rubreserine. Ehrenberg gave the empirical formula of rubreserine as $C_{13}H_{16}O_2N_2$. Salway confirmed this formula and found that eseroline absorbed two atoms of oxygen in forming rubreserine. In a study of the enzymic oxidation of eseroline in the presence of indophenol oxydase (cytochrome oxydase), Massart, Vandendriessche, and Dufait (12) also showed that

rubreserine was formed by the absorption of two atoms of oxygen These authors made the appropriate suggestion, although lacking experimental evidence, that the product might be an ortho quinone in accordance with the other known oxidation products of phenols formed by this enzyme Salway found that the empirical formula of eserine blue was $C_{17}H_{23}O_2N_3$, and that the substance formed a dihydrochloride with a formula $C_{17}H_{23}O_2N_3 \cdot 2HCl$ This author also observed that in the formation of the so called eserine brown, the end product formed when physostigmine stands in alkaline solution, five atoms of oxygen are absorbed per molecule of physostigmine Massart et al obtained a similar oxygen absorption when eseroline was completely oxidized in an enzymic system
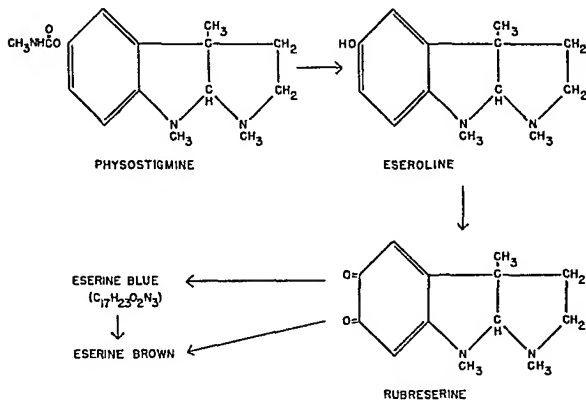
FIG 1 MECHANISM OF THE DECOMPOSITION OF PHYSOSTIGMINE

The series of reactions taking place in the decomposition of physostigmine are pictured in figure 1 The structure proposed for rubreserine and given in this figure is based upon the established empirical formula (10, 11) and the results presented in this paper

Preparation and chemical properties of the decomposition products of physostigmine The methods used in preparing the various products for this investigation are simpler than those of previous workers

Eseroline Five grams of physostigmine sulfate[3] was placed in a small continuous extractor and the air was displaced by a stream of oxygen free nitrogen A solution of 10% sodium hydroxide (30 ml), which had been boiled and cooled,

and then saturated with nitrogen, was then added from a dropping funnel to the physostigmine sulfate in the extractor and nitrogen bubbled through the suspension of physostigmine in sodium hydroxide for four hours. The reaction was completed by careful heating. The physostigmine was at this stage completely hydrolyzed to the phenolic eseroline, which was soluble in the alkaline solution. The alkaline solution was cooled, air-free ether was added through the dropping funnel, and the alkaline solution was extracted continuously for several hours. (The time necessary for the extraction was decreased in later runs by saturating the solution with carbon dioxide before extracting with ether.) The ether was evaporated under reduced pressure and the almost colorless, oily residue was left overnight in a vacuum desiccator containing sulfuric acid. On standing, the oil changed to a white solid which melted at 114–125°C. This solid was recrystallized twice from a mixture of benzene and petroleum ether and finally was washed with a small amount of cold ether. After the colorless crystalline material had been dried over sulfuric acid *in vacuo*, its melting point was 128°C., with slight softening at 124°. Salway (11) reported that the melting point of eseroline was 127–128°C.

A noteworthy property of eseroline is its ability to reduce silver nitrate to metallic silver, the eseroline solution turning red. Even in the crystalline state eseroline is difficult to keep. It turns pink in the air and when sealed in an ampoule it sometimes turns light green.

Rubreserine. A solution of 1 gram of physostigmine sulfate in water was made alkaline and extracted with ether. The ethereal solution of physostigmine was shaken with a small portion of 5% sodium hydroxide until the water layer had a strong pink color. The alkaline solution containing the rubreserine was quickly drawn off into a solution of sulfuric acid sufficiently acid to keep the mixture acid to Congo Red paper. The shaking of the ethereal solution with alkali was repeated several times until very little color appeared in the alkaline layer. The combined extract was made basic with 5% sodium hydroxide and rapidly extracted with chloroform. The chloroform extracts were combined, washed with water, and, without drying (rubreserine was strongly adsorbed by both calcium sulfate and calcium chloride and was very difficult to elute even with hot alcohol), the chloroform was evaporated *in vacuo*. A semi-solid, red material remained, and this, upon recrystallization from a mixture of chloroform and petroleum ether at −13°C., gave a red solid which, after drying *in vacuo* at 78°C. over sulfuric acid, melted at 132–133°C. Since another recrystallization had no effect on the melting point, this substance was considered to be the monohydrate of rubreserine as reported by Salway.

A sample of crystalline rubreserine which, when dried over solid potassium hydroxide, melted at 144–145°C., gave the following elementary analyses:[4]

	Calculated for $C_{15}H_{18}O_2N_2$	Salway (11) Reported
C = 66.25, 66.58, 66.08%	C = 67.2%	C = 67.5%
H = 7.30, 7.68, 7.33	H = 6.9	H = 7.2
N = 12.35, 12.79, 12.16	N = 12.1	N = 12.1

In the progress of this work some properties of this interesting compound have been noted which have not been stressed by previous workers. Rubreserine is yellow in concentrated hydrochloric and sulfuric acids. The substance can be extracted from sodium bicarbonate solution by chloroform, but the extraction is inefficient. It is soluble in most organic solvents, including ethyl acetate, but is insoluble in ether and only very slightly soluble in benzene. When dissolved in saturated barium hydroxide solution there is some green coloration intermediate to the formation of the yellowish-brown color of eserine brown, and this was taken as evidence of the formation of eserine blue from rubreserine. At pH 2.5 in a continuous extractor chloroform extracted rubreserine slowly and did not extract eserine blue from a mixture of the substances. A solution of rubreserine was changed to a slightly yellow solution by sodium hydrosulfite ($Na_2S_2O_4$) and the pink color returned when air was bubbled through the solution. Jobst and Hesse (1) had observed that H_2S, which was used to precipitate lead, discharged the pink color, which returned when the H_2S was removed. A color test for physostigmine reported by Mokragnatz (13), in which a purple color is obtained when a benzidine reagent and hydrogen peroxide are added to the alkaloid, gave no characteristic color when rubreserine was tested.

Eserine blue. Repeated evaporation of physostigmine in amounts of 10–100 mgm. with concentrated ammonium hydroxide on the steam bath in an uncovered watchglass resulted in a practically pure blue residue. Thick layers of this blue material appeared red by transmitted light. The residue was taken up in 0.1N HCl as a purple solution with an intense red fluorescence in ordinary light. The acid solution was made basic with sodium hydroxide and extracted with chloroform. The chloroform extract was blue by transmitted light and had an intense green fluorescence. By extracting the chloroform solution with 0.1 HCl and evaporating to dryness, a blue glassy solid was obtained. This was considered to be the dihydrochloride as reported by Salway. When the eserine blue was extracted from sodium bicarbonate solution by continuous extraction with chloroform and precipitated by the careful addition of petroleum ether, a green amorphous powder was obtained.

An electrometric acid-base titration of eserine blue gave a curve showing that the compound absorbed two equivalents of acid for its empirical formula and the curve had two inflections, at approximately pH 5 and 9. The solution of eserine blue showed a strong red fluorescence in acid solution below pH 4–5. Eserine blue in water was reduced by sodium hydrosulfite to a practically colorless substance; the blue color returned when air was bubbled through the solution. The reduced solution gave no color reaction with ferric chloride.

The determination of the molecular weight of eserine blue was planned, since only the empirical formula of $C_{17}H_{23}O_2N_3$ was given by Salway. However, the material proved to be insoluble in most common media, including camphor and ethylene dibromide, making a determination of this property inconvenient.

There is some difference of opinion about the mechanism of formation of eserine blue (11, 14). Since we were able to convert pure rubreserine to eserine blue by evaporation to dryness with NH₄OH, rubreserine is considered to be a precursor of the blue substance.

Eserine brown. Eserine brown is not a single chemical compound. It is the end product of the decomposition of physostigmine, and forms when physostigmine, eseroline, rubreserine, or eserine blue are heated in substance or in watery solution, or when they are allowed to stand in alkaline solution. Eserine brown is slightly soluble in water and alcohol, only very slightly soluble in acetone, and insoluble in ether. It dissolves to a reddish-brown in alkali and some of the brown material precipitates when the solution is acidified. As the decomposition proceeds further the material turns brownish-black and becomes entirely insoluble in the common solvents.

The melanin pigments obtained from adrenaline and tyrosine show the same solubility properties. These melanin pigments are probably structurally closely related, since all appear to pass through an intermediary indole-quinone compound in the process of their formation.

A solid amorphous preparation of eserine brown was obtained by evaporating to dryness a brown acidified solution of decomposed physostigmine and washing the residue by suspending the material in 0.1N HCl, centrifuging, and pouring off the supernate. The washing was repeated. A similar washing with acetone was repeated twice and the material dried over solid potassium hydroxide in a vacuum desiccator.

Adrenochrome: Adrenochrome has previously been prepared by Green and Richter (15) by an enzymic method. The following procedure has given a preparation of this relatively unstable material in a fairly pure, solid form.

One hundred milligrams of pure epinephrine[5] and 500 mgm. of dry silver oxide were suspended in 25 cc. of ethyl alcohol dried over drierite ($CaSO_4$) and agitated for one-half hour by shaking or by a stream of nitrogen. The resulting dark red solution containing the adrenochrome and the reduced silver and silver oxide was filtered through very fine filter paper. The filtrate was evaporated at room temperature under reduced pressure. This gave a solid red residue which was stable enough to stand sealed under nitrogen for one week with but slight decomposition. However, when this was exposed to air, the material turned to the insoluble melanin pigment with no soluble adrenochrome remaining after two days. The adrenochrome in bicarbonate Ringer solution at about 4°C. retained its red color for more than a week.

Adrenochrome in neutral solution is immediately decolorized by sodium bisulfite. Dilute solutions of adrenochrome and rubreserine are practically indistinguishable in color. However, when a dilute solution of rubreserine in bicarbonate Ringer is shaken with chloroform, the rubreserine is extracted and forms a lower orange-red layer, whereas adrenochrome is not extractable by chloroform under similar conditions. This may serve as a qualitative test.

2-Iodoadrenochrome. 2-Iodoadrenochrome was prepared from epinephrine by the method of Richter and Blaschko (16) and purified by washing with 2% acetic acid and acetone.

Evidence for the chemical structure of rubreserine proposed in figure 1. Massart

et al (12) suggested that rubreserine might be an ortho quinone   Chemical evidence in favor of their suggestion and supporting the structure which we propose in figure 1 was obtained by observing the green color imparted by ferric chloride to a reduced solution of rubreserine and the change to a reddish color on the subsequent addition of ammonia, a series of reactions considered typical for an ortho quinone   The characteristic test for quinones consisting of an immediate decoloration of the neutral solution by sodium bisulfite was given
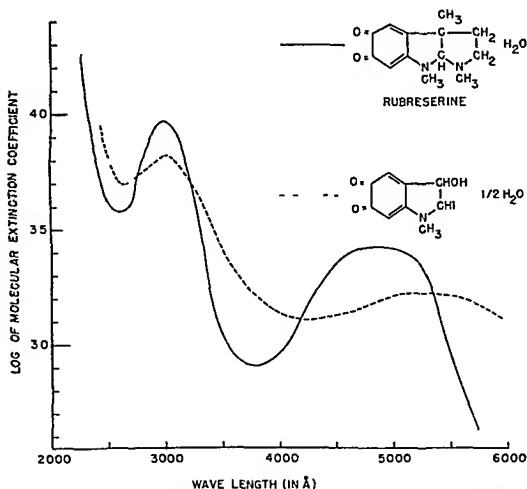
FIG 2 ABSORPTION SPECTRA OF RUBRESERINE AND OF 2 IODOADRENOCHROME
RUBRESERINE DISSOLVED IN WATER 2 IODOADRENOCHROME, IN AQUEOUS
DIOXANE

by rubreserine and also by the oxidation products of adrenaline, which are shown to be structurally related to rubreserine   The pink color returned on making the solution slightly basic

Since analogous ortho quinones containing the N methyldihydroindole group are known, it was suggested by Dr L F Fieser that a comparison of the ultra violet and visible absorption spectra of rubreserine with those of known com pounds would give further evidence for the structure of this compound   This type of evidence would show also which of the two possible ortho quinone struc tures the compound actually contained   The absorption spectra were obtained

through the courtesy of Dr. R. Norman Jones with an instrument (17) for ultraviolet absorption spectra which he modified by the use of a tungsten filament lamp as a light source for measurement at wave lengths greater than 4500Å.

In figure 2 the absorption spectra of rubreserine and an iodinated oxidation product of adrenaline are shown. Rubreserine was dissolved in water and the 2-iodoadrenochrome was dissolved in aqueous dioxane.

The absorption spectrum of adrenochrome is shown in figure 3. Since the sample used was not entirely soluble (some melanin had formed on standing)
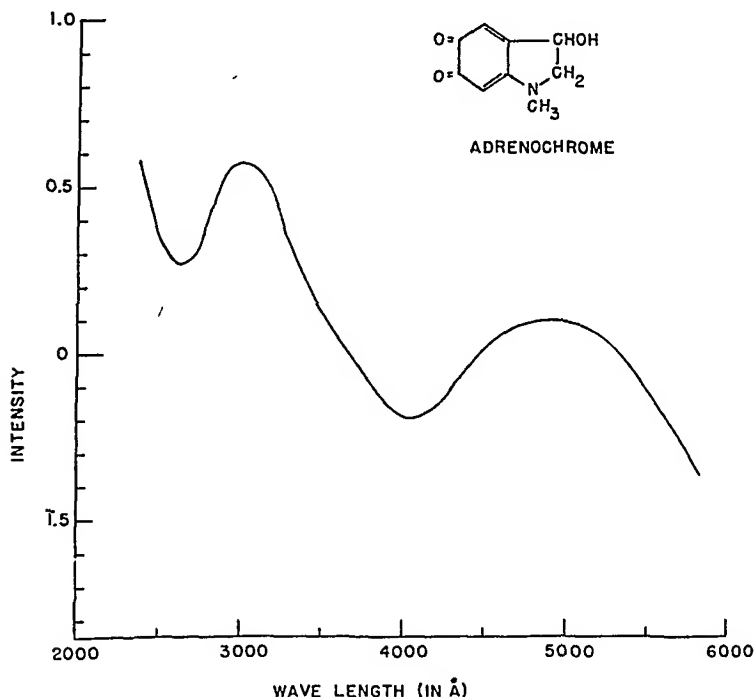
FIG. 3. ABSORPTION SPECTRUM OF ADRENOCHROME. SOLVENT, WATER. INTENSITY = LOG OF OPTICAL DENSITY $\left(\text{LOG} \dfrac{I_0}{I} \right)$ × DILUTION FACTOR

the graph was drawn on an intensity scale depending on fractional dilution rather than absolute concentration. The absorption spectrum was obtained with water as the solvent.

The absorption curves of these compounds are of the same type, and the positions of the maxima at about 3000Å are practically coincident. This absorption peak at 3000Å is significant in view of the ultraviolet absorption bands of physostigmine reported by Brustier (18), who found an absorption band at 2783.8–3370.9, with its peak at about 3000Å. The absorption of the com-

pounds in the visible range does not agree as well, but the wave length of maximum absorption of adrenochrome in this region is almost identical with that of rubreserine   In this connection it is interesting that Mazza and Stolfi (19) reported that visible absorption of the pigment hallochrome, which is N methyl 2 carboxy dihydroindole 5,6-quinone, shows a maximum at 4500Å, which is in agreement with that of 2 iodoadrenochrome, an analogous compound with an iodine atom in place of the similarly heavy carboxyl group

The red pigment obtained by Arnow (20) from human red hair with an absorption maximum at about 5800Å may be similar in structure to the dihydro indole quinones reported in this paper   This would certainly be of interest in the general problem of pigment formation in the body which is thought to make melanin substances from tyrosine or adrenaline

The anticholinesterase activity of rubreserine and eserine blue on the horse serum enzyme or in biological preparations is described in detail in the previous paper of this series (3)

Waelsch and Rackow (21) reported that some of their preparations of adrenochrome markedly inhibited serum cholinesterase *in vitro* in concentrations as low as 10^{-4} molar (approx 1 50,000)   In some preliminary studies we found no evidence for inhibition of cholinesterase using pharmacological methods   Thus, the leech muscle was not sensitized to acetylcholine below a concentration (1 1,000) which in itself caused activity in the leech muscle, nor was the frog's rectus abdominis sensitized   The isolated frog heart responded to adrenochrome in concentrations as high as 1 10,000 with very slight increases in rate and amplitude   In the isolated rabbit gut a concentration of 1 300,000 was sufficient to suppress the spontaneous activity and this action was reversible by washing

Although there is striking similarity in the chemical structures of rubreserine and the oxidation product of epinephrine, none of our results are indicative of cholinesterase inhibition by adrenochrome in the biological systems studied

SUMMARY

1 Methods are described for the preparation of eseroline, rubreserine, eserine blue, eserine brown, and adrenochrome

2 Rubreserine is shown to contain a substituted 2,3 dihydroindole 5,6 quinone group and thus to be structurally related to adrenochrome, the oxidation product of epinephrine

3 The absorption spectra in the range 2200–6000Å of rubreserine, 2 iodo-adrenochrome, and adrenochrome are reported   The spectra are similar in that each has a maximum at about 3000Å and another absorption peak in the range of 4900 5400Å

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THE RELATION BETWEEN CHEMICAL STRUCTURE AND PHYSIOLOGICAL DISPOSITION OF A SERIES OF SUBSTANCES ALLIED TO SULFANILAMIDE[1]

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The success of sulfanilamide and its derivatives in the field of bacterial chemotherapy has stimulated investigators to inquire into the biological mechanisms which are involved in their specific action (1, 2, 3). These studies deal with the relation of general chemical structure to specific physico-chemical properties of the compounds and with the relation of the latter to antibacterial activity. It must be appreciated, however, that the in vivo activity of such an agent is also dependent upon those properties of the organic complex which condition its physiological disposition. The latter properties determine the ability of a compound to reach a locus of action in the body, the concentration which it achieves at that locus, and the duration an adequate concentration is maintained. A fair amount of information is now available on the absorption, distribution and excretion of many commonly used sulfonamides (4, 5, 6, 7, 8, 9, 10, and others). However, the number of compounds studied is inadequate and the experimental preparations too diverse for general conclusions to be reached on the relation between the chemical structure of compounds in this series and the details of their physiological disposition.

The present report contains the results of investigations on the distribution and excretion of thirty compounds related to sulfanilamide. Observations have been made on the distribution of each of these in the body as a whole, as well as in specific tissues, and on the ability of each to pass the normal blood-brain barrier, to participate in a specific cellular activity as represented by renal tubular excretion and reabsorption, and, finally, to form reversible combinations with the non-diffusible constituents of plasma. An attempt is made to correlate these properties with the chemical structure of the compounds.

EXPERIMENTAL Cats, averaging 2 5 kilograms, were used in the distribution studies Light anesthesia was produced by the intraperitoneal administration of minimal amounts of nembutal The abdomen was opened, both kidneys isolated, and the renal pedicles securely ligated The abdomen was then closed by sutures An exactly weighed amount of the sulfonamide under examination was dissolved in warm saline and administered by intravenous injection. The body temperature of the animals was maintained at a normal level in the short experiments by the use of a heated blanket and, in the longer experiments,

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by the use of a warmed cage. The animals in the latter experiments were usually conscious and in good condition in 24 hours.

Blood samples were drawn by intracardiac puncture one hour after the administration of the sulfonamide and just prior to the termination of the experiment. Blood was drawn, using oxalate as an anticoagulant, and the samples centrifuged immediately. The separated cells and plasma were refrigerated until they were prepared for analysis. The animals were sacrificed by exsanguination. Cerebrospinal fluid was withdrawn from the exposed cisterna by a syringe and needle. Samples of tissue were then removed and placed in covered weighing dishes. The procedure, from the last blood sample to the removal of the tissue samples, was usually accomplished in less than 15 minutes.

The excretion studies were performed with three normal, well-trained female dogs. The dogs were maintained on a standard dog chow diet, but were not fed on the morning of the experiment. Each experiment was started by the administration of 40–50 ml. of water per kg. by stomach tube and the subcutaneous injection of 200 mg. per kg. of creatinine. These procedures assured an adequate diuresis and a plasma creatinine of 15–20 mg. per cent which were sustained throughout the period of observation. The sulfonamide was administered subcutaneously at the same time as the creatinine, in a dosage calculated to produce a plasma concentration of about 5 mg. per cent. Each experiment consisted of 3 or 4 urine collection periods of from 10–15 minutes each, with a blood drawn at the mid-period. The first period was started 45 minutes after the administration of the water, creatinine and the sulfonamide. The experiments were planned so that each dog had a rest period of not less than three days between observations.

METHODS. Sulfanilamide and its related compounds were determined in protein-free filtrates of tissues and the various biological fluids by the method of Bratton and Marshall (11). A departure from the recommended procedure was found to be necessary in several instances. Metanilamide, sulfanilic acid and the N^4-sulfanilyl sulfanilic acid required additional hydrochloric acid for complete diazotization. Orthanilamide required a longer time than is usual for the completion of both diazotization and coupling.

The plasma was prepared for analysis by the precipitation of plasma protein with tri-chloracetic acid at a dilution of 1–40. The red blood cells were first hemolyzed in water and the protein-free filtrates then prepared in a similar manner. The tissue samples were first weighed and then ground with washed silica, the tissue protein being precipitated at a high dilution of the tissue by the use of trichloracetic acid. They were extracted for thirty minutes with gentle agitation, then centrifuged, and the supernatant fluid cleared by filtration. The urines, in the excretion studies, were diluted with 3 per cent trichloracetic acid so that the final concentration of the sulfonamide was approximately the same as that obtaining in the plasma filtrates.

Recoveries were performed on each of the sulfonamides in the presence of water, plasma red blood cells and tissues. All compounds were recoverable from plasma to the extent of 96 per cent or better when the plasma was precipitated in a dilution of not less than 1–40 Recoveries of some of the sulfonamides from erythrocytes were not very satisfactory. The acyclic derivatives of sulfanilamide and the simple allied compounds were recoverable to an extent of 90 per cent or better. Recoveries of the N^1-isocyclic derivatives were poor, varying from 50 to 80 per cent of the amount added. The heterocyclic derivatives yielded recoveries of 90 per cent or better for sulfapyridine, sulfadiazine, and sulfamethyldiazine but only 70 per cent for sulfathiazole and sulfamethylthiazole. Recoveries from all other tissues were better than 90 per cent with the exception of orthanilamide and the N^1-carboxyphenyl derivatives of sulfanilamide. The distribution ratios in Tables 3 and 4 which are marked with an asterisk contain a correction for the incomplete recovery of the sulfonamide.

Creatinine was determined by a modified Folin procedure on tungstic acid filtrates of plasma prepared to a 1–20 dilution, and on urine diluted so that the creatinine concentration was essentially that of the plasma filtrate (12).

The extent of binding of each of the sulfonamides on the non-diffusible constituents of

plasma was determined by dialysis against isotonic salt solution at pCO₂ 38 mm , T 37°C Cellophane membranes were used in most cases although a few determinations were made with parlodion Normal dog plasma was used, and the bound fraction in each determination was corrected to that which would obtain at a plasma albumin of 4 00 grams per 100 ml of plasma An examination of thirteen of the sulfonamides was also made using cat plasma The extent of the binding of the compounds studied was in the same order of magnitude as was observed with normal dog plasma

RESULTS[2] Tables 1 and 6 present the essential data of experiments which define the distribution of sulfanilamide in the cat and its renal excretion by the dog These are presented in detail since they illustrate the two general types of experiments used in these studies and the methods used in the calculation of the data

Studies on distribution The data contained in table 1 are derived from an experiment which examines the distribution of sulfanilamide two hours after its intravenous administration The experiment is quite typical of the series as a whole insofar as distribution equilibrium appears to be approximated in one hour since the plasma concentration of sulfanilamide at that time is much the same as

TABLE 1

An experiment which examines the distribution of sulfanilamide in the cat

9 25—Nembutal, kidneys ligated   10 00—27 8 mg /kilo sulfanilamide I V (wt 2 05 kilos)   11 00—B₁ plasma—2 89 mg per cent   12 00—B₂ plasma—2 68 mg per cent

Tissue samples   Calculated volume of distribution = 3060 ml or 96 5 per cent body weight

PLASMA	CONCENTRATION MG /100 ML (OR GMS)				RATIO	TISSUE CONCENTRATION (MG /100 G) / PLASMA CONCENTRATION (MG /100 ML /0 93)			
	RBC	Muscle	CSF	Brain		RBC	Muscle	CSF	Brain
2 68	4 32	3 08	2 11	2 01		1 50	1 07	0 73	0 70

at the end of the two hour interval   These data indicate that sulfanilamide diffuses readily into all tissues and is localized in the cells of many (5, 13)   This conclusion stems from the finding that the volume of distribution of sulfanilamide is in excess of the water content of the body and that the ratio of the sulfanilamide concentration in any tissue to its concentration in plasma water is greater than is true for water itself (14)   Were sulfanilamide freely diffusible throughout the body and not specifically localized in any tissue, its volume of distribution would be in the order of 65 per cent of the body weight and the tissue/plasma distribu

tion ratios would be considerably lower than those which were observed. On the other hand, if the sulfanilamide were largely retained in an extracellular position it would have a volume of distribution in the order of magnitude of 25-30 per cent of the body weight (15, 16) and its distribution ratios would be similar to those of sodium or chloride. The latter figure, in the case of muscle, would be in the range of 0.11-0.12 (16). The presence of sulfanilamide in a relatively high concentration in the cerebrospinal fluid is in keeping with the observations of others (5) and indicates that the compound penetrates the blood-brain barrier quite freely. The absence of a concentration which is identical to that of plasma is due in part to the factor of plasma binding and possibly in part to the rate of turnover of cerebrospinal fluid so that a complete equilibrium is not reached.

It will be of some value, before proceeding further, to examine some of the potentialities and limitations of the experimental preparation. The ligation of the renal pedicles makes available a reasonably normal animal, in which the compound under study is contained in a closed system. It is to be expected that a dynamic equilibrium will be established between the concentration of the substance in the fluid of reference, i.e., plasma water, and its concentration in the other compartments of body fluid. The elimination of renal excretion may be expected to facilitate the prompt attainment of diffusion equilibrium and to stabilize the equilibrium concentrations which are achieved.

A change in the plasma concentration of a substance subsequent to the attainment of an initial equilibrium under these conditions is an indication that a change in the chemical structure of the compound has occurred, or, that there has been a change in the biological activity of some of the large groups of cells in the body. The latter of these two factors need not complicate the interpretation of the data in such a situation since it may be excluded by properly planned experimental procedures (c.f. 13). It is to be expected that the free aryl amino groups of the sulfonamides will become progressively conjugated in the cat. This change in chemical structure will produce a general lowering of the concentration of the parent substance in the plasma and in all tissues. It is not expected to change the plasma/tissue distribution ratios of the parent compound since such conjugated products are excluded in the chemical analyses. However, insofar as a chemical modification does occur, it would be surprising if the resulting substance were characterized by a distribution in the body which is identical to that of the parent compound. It should be possible, then, to use the preparation in a limited fashion in the exploration of the extent to which a change in chemical constitution occurs subsequent to the introduction of a compound into the body, to follow the rate of the change, and, in certain cases, to obtain some indication of the nature of the chemical modification. The use of the dog in these experiments would have simplified the experimental conditions since this species does not acetylate compounds of the present series to any considerable extent.

The experiments which have been used to describe the distribution of this series of compounds in the body are limited to those of two hours' duration. This interval is ample for the establishment of a stable diffusion equilibrium within the body, but is too short for an extensive change in the chemical struc-

ture of the compound under investigation. It must be appreciated, in considering the data presented below, that a precise definition of the distribution of each compound has not been achieved. The magnitude of such a problem in the case of a single compound is not small. Reference to the studies of recent years on the distribution in the body of such substances as sodium, potassium and the halogens (16) will serve to fortify this view. Moreover, it must be appreciated that the data which indicate that a compound enters a cell or organ yield no information on the state of the material so contained, or, on the actual concentration ratio of the substance across the cell membrane, i.e., the concentration relationships in extra- and intracellular water. It is not unlikely that a considerable proportion of the specific solute within a cell reacts with cell constituents in such a manner that a portion is removed from free solution in intracellular water.

Tables 2, 3 and 4 present a summary of the distribution data on the compounds examined. Each value represents the average of at least two experiments.

The distribution data on a series of acyclic derivatives of sulfanilamide and a series of closely allied compounds are summarized in table 2. It is apparent that the addition of non-polar groups in the N^1 position of the sulfanilamide molecule does not drastically affect the distribution of the compound as compared to sulfanilamide itself. The resulting substances penetrate cell membranes of all tissues and, in addition, are localized within the contents of most cells. The N^1-methyl and the N^1-ethyl appear to enter cerebrospinal fluid more freely than sulfanilamide itself. However, the latter substance passes the blood-brain barrier so readily that the difference is not striking. The N^1-hydroxy, N^1-amino, and the N^1-ethanol derivatives unquestionably show an impairment in this ability which is quite striking in the case of the sulfanilyl guanidine. The data on the N^1-hydroxy are not very satisfactory since a fairly rapid reversion to sulfanilamide is to be expected and consequently an eventual distribution in the body which is characteristic of the latter substance.

However, when strongly acidic groups are added in the N^1 position quite a different finding is the rule. The N^1-acetyl derivative of sulfanilamide and sulfanilyl glycine have a distribution which is quite similar to that of sulfanilic acid, as given below. This circumstance presumably follows from the fact that they are organic acids with a strength of the same order of magnitude as sulfanilic acid. This appears to be reflected in the degree to which all three compounds are excluded from cerebrospinal fluid and to some extent from cellular water in general. However, the correlation between overall distribution and acidic strength is not very precise, as may be seen from an examination of the last two columns of table 2. Inspection of specific tissues, particularly muscle vs. cerebrospinal fluid, lends support to the belief that the distribution across the normal blood-brain barrier is determined in part by unspecified but general characteristics of a molecule as well as by the charge carried on a specific group or its tendency to dissociate a hydrogen ion. An example of the operation of the former factor in the series of compounds is found in the low CSF/plasma ratio of sulfanilyl guanidine.

The data on paraminobenzoic acid are particularly worthy of note. One would

TABLE 2

Summary of observations on the distribution in the cat of a series of N¹-acyclic derivatives of sulfanilamide and a series of simple compounds closely allied to sulfanilamide

$$H_2N\langle\hspace{1cm}\rangle SO_2NH{-}R$$

—R	TIME	RATIO: $\dfrac{\text{TISSUE CONCENTRATION (MG./100 G.)}}{\text{PLASMA CONCENTRATION (MG /100 ML /0.93)}}$								VOLUME OF DISTRIBUTION	pKa
		CSF	Brain	RBC	Lung	Liver	Pancreas	Muscle	Nerve	% body weight	
—H	2	0.68	0.73	1.30	1.07	1.37	1.03	1.07	0.62	98.2	10.43
	24	0.83	0.78	1.08	1.06	1.15	1.02	1.06	0.94		
—CH₃	2	0.77	0.85	1.14	1.02	1.29	0.96	1.02	0.95	91.7	10.77
	24	0.76	0.83	1.12	1.00	1.19	1.01	0.98	0.90		
—CH₂CH₃	2	0.89	0.92	1.23	1.06	1.52	1.20	1.11	0.93	102.0	10.70
	24	0.80	0.90	0.94	1.14	1.39	1.15	1.08	0.89		
—OH	2	0.58	0.60	1.33	1.08	1.20	0.97	1.07	0.82	99.0	
	24	0.99	0.78	1.11	1.01	1.01	0.91	1.01	0.83		
—NH₂	2	0.43		0.87						72.5	10.70
—CH₂CH₂OH	2	0.30	0.21	0.67	0.82	1.35	0.71	0.70	0.51	67.2	10.94
	48	0.07	0.07	0.52	0.57	0.67	0.37	0.18	0.65		
—C=NHNH₂	2	0.09	0.08	0.93	1.17	1.29	1.16	0.51	0.62	78.6	*
	48	0.30	0.48	1.06	1.14	1.19	1.03	1.07	1.00		
—CH₂COOH	2	0.03	0.04	0.22	0.50	0.55	0.30	0.16	0.35	29.9	3.52
	24	0.05	0.03	0.49	0.52	0.72	0.32	0.20	0.63		
—COCH₃	2	0.05	0.06	0.76	0.54	0.59	0.45	0.37	0.46	55.5	5.38
	48	0.88	0.79	1.09	1.02	1.04	1.05	1.09	0.99		
Simple Allied Compounds											
$H_2N\langle\rangle SO_2NH_2$	2	0.76		1.08						103.0	10.12
$H_2N\langle\rangle SO_2NH_2$	2	0.71	0.84	1.08	1.08	1.21	0.97	1.00	0.65	92.3	10.12
	24	0.83	0.91	1.08	1.07	1.30	0.95	0.98	0.93		
$CH_3COHN\langle\rangle SO_2NH_2$	2	0.27	0.15	1.28	0.89	1.09	0.86	0.97	0.48	84.2	
	24	0.28	0.20	1.11	0.75	0.74	0.71	0.72	0.59		

TABLE 2—Continued

—R	TIME	RATIO $\dfrac{\text{TISSUE CONCENTRATION (MG /100 G)}}{\text{PLASMA CONCENTRATION (MG /100 ML /0 93)}}$								VOLUME OF DISTRIBUTION	pKa
		CSF	Bn a	RBC	Lung	Liver	Pancreas	Muscle	Nerve		
H₂N⟨⟩SO₂OH	2	0 03	0 03	0 65	0 47	0 29	0 24	0 11		*% body we tht* 28 7	3 20
	24	0 03	0 04	0 62	0 57	0 36	0 29	0 13			
H₂N⟨⟩COOH	2	0 64	0 32	0 47	0 57	1 66	0 29	0 40	0 42	51 4	4 68
	48	0 03	0 10	0 55	0 61	0 83	0 35	0 19	0 58		

* Very weak

expect, from inspection of the molecule and the pKa value of the carboxyl group, that its distribution would not be very different from the distribution of sulfanilylglycine or, perhaps, that its distribution would be midway between the latter compound and the N^1 acetyl derivative of sulfanilamide The correlation is not too bad in the case of the volume of distribution and the tissue/plasma distribution ratio of muscle However, it diffuses into cerebrospinal fluid quite as easily as sulfanilamide itself It should be emphasized, taking paramino benzoic acid as a specific sample, that a tissue/plasma distribution ratio is not necessarily an expression of the concentration ratio across the cell membrane Assuming that this substance is of importance to the economy of the living cell, it would be surprising were it not localized within the cell in a manner which makes some of the cellular paraminobenzoic acid unavailable for conditioning its rate of diffusion

It is also of some interest to note that the N^4 acetyl derivative of sulfanilamide has less restriction imposed on its distribution in the body than when the acetyl is in the N^1 position A simple explanation of this circumstance may be that the N^4 acetyl is the weaker acid However, the divergence in distribution of these compounds in the body as a whole and their distribution across the blood brain barrier suggests that other factors than acidic strength may operate in restricting the diffusion of these compounds in the body

A summary of the distribution of twelve isocyclic derivatives of sulfanilamide is given in table 3 Again, the presence or absence of a reasonably strong acidic group appears to be important in determining the ability of a substance to penetrate cell membranes in general, as evidenced by the volume of distribution of a compound in relation to the value of its pKa However, the situation with respect to these compounds is not as simple as would appear from the data A modification of the molecular structure of sulfanilamide to include a substituted or non substituted additional benzene ring throws into prominence the ability of the non-diffusible constituents of plasma to form reversible combinations with the resulting compounds (see Tables 7, 8 and 9) It would be apparent, were a

TABLE 3

Summary of observations on the distribution in the cat of a series of N^1-isocyclic derivatives of sulfanilamide

$$H_2N\langle\ \rangle SO_2NH\langle\ \rangle$$
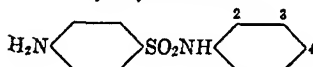
SUBSTITUENT	TIME	RATIO: $\dfrac{\text{TISSUE CONCENTRATION (MG./100 G.)}}{\text{PLASMA CONCENTRATION (MG./100 ML./0.93)}}$								VOLUME OF DISTRIBUTION	pK_a
		CSF	Brain	RBC	Lung	Liver	Pancreas	Muscle	Nerve	% body weight	
	2	0.18	1.06	0.89*	1.20	2.46	1.72	0.80	0.75	125.0	9.60
	24	0.03	0.14	0.27	0.64	0.79	0.42	0.22	0.37		
—,—CH₂CH₂OH†	2	0.40	0.65	0.91*	1.00	2.32	1.07	0.96	0.86	127.0	
	24	0.13	0.27	0.37	0.77	1.82	1.19	0.44	0.50		
4—NH₂	2	0.33	0.51	1.09	1.43	2.54	1.35	1.15	0.79	124.0	10.22
	24	0.33	0.50	0.84	1.19	2.54	0.89	0.87	0.99		
2—SO₂NH₂											
3—SO₂NH₂	2	0.14	0.19	2.17*	1.44	2.58	1.71	0.94	0.84	146.0	8.23
	24	0.13	0.37	1.16	1.09	2.44	1.05	0.85	0.71		
4—SO₂NH₂	2	0.07	0.15	2.78*	1.77	3.14	1.98	1.23	0.84	165.5	7.85
	48	0.12	0.29	0.90	1.22	1.70	1.11	0.78	1.18		
2—COOH	2	0.01		0.66*						31.6	3.85
3—COOH	2	0.01	0.03	0.32*	0.31	0.45	0.17	0.07	0.19	23.0	4.10
	24	0.03	0.07	0.25	0.60	0.57	0.21	0.10	0.36		
4—COOH	2	0.01	0.04	0.41*	0.49	0.51	0.25	0.15	0.28	36.2	4.05
	24	0.02	0.10	0.58	0.66	0.71	0.42	0.23	0.77		
2—SO₂OH	2	0.01	0.06	0.46*	0.49	0.47	0.38	0.10	0.17	23.9	3.40
	24	0.01	0.09	0.19	0.50	0.61	0.44	0.11	0.41		
3—SO₂OH	2	0.01	0.04	0.16*	0.37	0.28	0.23	0.08	0.26	18.8	3.35
	24	0.01	0.05	0.22	0.54	0.27	0.25	0.10	0.29		
4—SO₂OH	2	0.02	0.04	0.48*	0.71	0.51	0.31	0.14	0.51	35.7	3.40
	24	0.03	0.05	0.31	0.67	0.48	0.38	0.14	0.68		

* Corrected for poor recovery.
† N^1 phenol N^1 ethanol sulfanilamide.
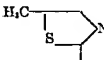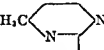
correction made for this factor, that the tissue concentrations of the strongly acidic compounds of this series are somewhat in excess of that which would obtain were they restricted to an extracellular position, and that the concentrations of

the others of this series are exceedingly high as compared to their concentration in extracellular water   The data on the N^1 phenyl, the N^1 amino phenyl and the N^1 sulfonamido phenyl derivatives indicate a very extensive localization of each within the cells   However, the distribution of these substances across the blood brain barrier emphasizes the specific characteristics of the barrier which

TABLE 4

Summary of observations on the distribution in the cat of a series of N^1 heterocyclic derivatives of sulfanilamide

$$H_2N\langle\bigcirc\rangle SO_2NH{-}R$$
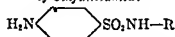
—R	TIME	RATIO								VOLUME OF DISTRIBUTION	pKa
		TISSUE CONCENTRATION (MG /100 G) / PLASMA CONCENTRATION (MG /100 ML /0 93)									
		CSF	Bra a	RBC	Lung	Liver	Pancreas	Muscle	Nerve	*% body wt ght*	
(structure: sulfapyridine)	2	0 62	0 80	0 83	0 97	1 33	0 93	0 91	0 68	82 5	8 44
	24	0 47	0 47	0 65	0 86	1 09	0 74	0 60	0 57		
(structure: sulfathiazole)	2	0 10	0 14	0 83*	0 71	1 13	0 60	0 54	0 32	58 5	7 12
	24	0 28	0 35	0 68	0 75	1 39	0 63	0 57	0 61		
(structure: methyl sulfathiazole)	2	0 13	0 16	0 57*	0 49	1 19	0 46	0 46	0 35	51 4	7 80
	24	0 20	0 35	0 47	0 63	1 46	0 59	0 43	0 61		
(structure: sulfadiazine)	2	0 31	0 21	0 53	0 60	0 63	0 44	0 45	0 38	46 0	6 48
	48	0 81	0 70	0 78	0 83	0 90	0 70	0 74	0 74		
(structure: sulfamerazine)	2	0 38	0 35	0 45	0 56	0 76	0 47	0 39	0 50	45 8	7 06
	24	0 64	0 43	0 64	0 67	1 22	0 66	0 59	0 55		

* Corrected for poor recovery

normally sets cerebrospinal fluid and the interstitial fluid of the brain apart from the interstitial fluid of the other organs of the body

The data on the distribution of a series of N^1 heterocyclic derivatives of sulfanilamide are summarized in table 4   The degree to which each of these is bound on plasma protein is also important in considering the data on their distribution   The order of magnitude for each of the five is 30 per cent for sulfa pyridine, 60 per cent for sulfathiazole, 80 per cent for the 4 methyl sulfathiazole (sulfamethylthiazole), 20 per cent for sulfadiazine, and 40 per cent for the 4 methyl sulfadiazine (sulfamerazine)   It is possible to conclude, with this infor

mation, that localization occurs within the tissue cells in the case of sulfapyridine, sulfathiazole and sulfamethylthiazole, whereas sulfadiazine and sulfamerazine are distributed more as if they freely penetrate most cell membranes, but are not specifically localized within the cells. The spread in the dissociation constants in the series is not great, and over this limited range there does not appear to be any important correlation between them and the distribution of the compounds.

It is apparent from these data that the normal blood-brain barrier has specific characteristics which set cerebrospinal fluid and interstitial fluid of the brain apart from the interstitial fluid of the other organs of the body. The ability of the compound to enter the former compartment of fluid appears to depend not only upon its acidity, i.e., its tendency to dissociate a hydrogen ion, but also upon the other more general properties of the molecule related to its molecular configuration. The latter property is particularly apparent in the case of the isocyclic derivatives of sulfanilamide of which the N^1-phenyl sulfanilamide and the sulfanilyl sulfanilamide are good examples. Both of these compounds have a relatively high pKa and both are widely distributed in the tissues of the body as a whole. However, they appear to be unable to enter cerebrospinal fluid to an appreciable degree. Further examples of the operations of this factor are found in the data on the distribution of both the acyclic and heterocyclic derivatives. Many of these appear to be impaired in their ability to cross the blood-brain barrier out of proportion to their acidic strength as represented by their pKa values. It may be noted that the brain/plasma ratios follow very closely those of the cerebrospinal fluid/plasma ratio. This finding is in keeping with the view that the chemical composition of the interstitial fluid of brain tissue is more similar to that of cerebrospinal fluid than to that of plasma water (17, 18, 19, 20 and others). Similar findings were observed with many of the compounds when the spinal cord was examined. However, the data on the latter tissue are too few to warrant separate presentation.

As noted above, a change in the distribution of a compound in the body subsequent to the attainment of an initial equilibrium is probably indicative of a change in the chemical constitution of the administered material. An example of such a change in distribution is given in table 5. These data summarize the experiments on the distribution of the N^1-ethanol derivative of sulfanilamide. The initial distribution of this compound in the body is quite similar to that of sulfanilamide (table 2). However, a change in distribution is already apparent at 5 hours and this is almost complete at 24 hours. The distribution at 48 hours is quite similar to that of sulfanilylglycine. It is not possible to conclude from these data that the chemical transformation is in fact an oxidation of the alcohol to the acid, although this does seem quite possible. A somewhat similar change was observed in the case of N^1-ethanol N^1-phenyl sulfanilamide (table 3). Other striking changes are found in the data on the N^1-acetyl sulfanilamide and sulfanilyl guanidine. The latter two changes suggest a simple loss of the N^1 substituent. However, the most important implication of the data as a whole is that many compounds in each of the general classes studied undergo some chemical modification exclusive of the acetylation of the N^4-nitrogen.

Studies on renal excretion The overall renal excretion of each compound was studied in experiments such as that summarized in table 6 The experiments made use of experimental techniques which permit a general description of the renal mechanisms involved in the excretion of a compound The underlying

TABLE 5

A study of the change in distribution in aryl amine subsequent to the administration of N¹ ethanol sulfanilamide

The data for the 2, 24 and 48 hour time intervals represent the mean of two or more experiments The data on sulfanilyl glycine is presented for comparison with the distribution obtaining 48 hours subsequent to the administration of N¹ ethanol sulfanilamide

COMPOUND	TIME	RATIO TISSUE CONCENTRATION (MG /100 G) / PLASMA CONCENTRATION (MG /100 ML /0 93)								VOLUME OF DISTRIBUTION
		CSF	Brain	RBC	Lung	Liver	Pancreas	Muscle	Nerve	*% body wt g/t*
N¹ ethanol sulfanilamide	2	0 30	0 21	0 67	0 82	1 35	0 71	0 70	0 51	67 2
	5	0 30	0 23	0 55	0 68	1 31	0 65	0 58	0 53	
	24	0 11	0 12	0 40	0 50	0 79	0 35	0 20	0 52	
	48	0 07	0 07	0 52	0 57	0 67	0 37	0 18	0 65	
Sulfanilyl glycine	2	0 03	0 04	0 22	0 50	0 55	0 30	0 16	0 35	29 9

TABLE 6

An experiment which examines the renal excretion of sulfanilamide

Dog K

— 45–800 ml water per os Creatinine 200 mg /k subcut sulfanilamide 75 mg /k subcut — 00—Bladder emptied 4 collection periods 15 minutes each Bloods at mid period

PERIOD	CREATININE		GLOMERULAR FILTRATION RATE	SULFANILAMIDE					CLEARANCE RATIO	EXCRETION RATIO
	Plasma	Excretion		Plasma	Plasma (filterable)	Filtered	Excreted	Reabsorbed		
	mg /ml	*mg /m n*	*ml /m n*	*mg /ml*	*mg /ml*	*mg /m n*	*mg /m n*	*mg /m n*		
1	0 144	6 82	47 4	0 039	0 035	1 66	0 39	1 27	0 21	0 23
2	0 150	6 92	46 1	0 045	0 041	1 89	0 44	1 45	0 21	0 24
3	0 150	7 32	48 7	0 051	0 045	2 19	0 55	1 64	0 23	0 25
Mean			47 0						0 22	0 24

principles are simple Glomerular filtration rate is measured following the administration of creatinine, the rate of glomerular filtration in milliliters per minute being equal to the dividend of the milligrams of creatinine excreted per minute and the plasma concentration of creatinine in milligrams per milliliter (21, 22, 23, 24) Glomerular filtration rate so determined was calculated to be 47 4 milliliters per minute in the first period of the experiment summarized in table 5

The data on the excretion of sulfanilamide, obtained simultaneously, may be manipulated as follows: The plasma concentration of sulfanilamide during this period was 0.039 mgm. per ml., 90 per cent of which is in a filterable form. At a glomerular filtration rate of 47.4 ml. per minute, it may be calculated that 1.66 mgm. of sulfanilamide was filtered each minute. However, since the concurrent renal excretion accounts for only 0.39 of the 1.66 mgm. filtered each minute, one must conclude that 1.29 mgm. of filtered sulfanilamide were reabsorbed in the renal tubules. The calculation throws little light on the mechanism of the reabsorptive process. However, the ratio of the amount filtered to the amount excreted, in this case 0.23, yields a figure which is characteristic of the excretion of the compound by the average nephron under the conditions of the experiment. This ratio, which we designate the *excretion ratio*, is a useful datum in the study of the renal excretion of a series of allied compounds.

Another ratio which may be calculated from the data, and which does not include a correction for the factor of plasma binding, is the sulfonamide/creatinine *clearance ratio*. The latter ratio is important in the definition of the overall renal excretion rates of compounds, but is less useful in indicating the mechanism by which such excretion is accomplished.

The conclusion that sulfanilamide is reabsorbed to a considerable extent is contingent on the demonstration that essentially all of the plasma sulfanilamide is filterable at the glomerulus. Such a circumstance does not hold for many of the compounds which have been examined in the present study. However, the excretion ratio, containing as it does a correction for the factor of plasma binding, makes available a corrected ratio which, theoretically, is a rather precise expression of the degree to which a substance is reabsorbed or actively excreted by the renal tubules. It has two limitations. The ratio gives limited information on the nature of the physiological mechanisms involved and it is quite sensitive to errors in the case of substances which are extensively bound on plasma protein.

Precise information on the underlying mechanisms which are responsible for renal tubular reabsorption or excretion can be obtained by varying the experimental procedures. However, as is the case of studies on distribution of compounds, a quantitative definition of the renal mechanisms involved in the excretion of a single substance is a task of major proportions. This viewpoint can perhaps be best appreciated by reference to studies on the excretion of inulin and creatinine (21, 22, 23, 24), urea (25, 26), glucose (12), and phenol red (27). These studies were also performed in the dog and in the case of each substance define its excretion in terms of the discrete renal mechanisms which are involved. The data detailed below are in the nature of a general survey of the excretion of compounds related to sulfanilamide. Extraneous factors were minimized by adhering to rigidly standardized experimental conditions. These include a state of moderate diuresis, and a plasma concentration for the substance under examination in the order of magnitude of 5.0 mgm. per cent. All observations were made within 90 minutes after the administration of the compound.

It may be accepted in the following that an excretion ratio of 1.0 is indicative of a situation wherein the renal tubules do not participate in the excretion of a

compound to a measurable extent   As the ratio falls progressively below 1 0, it is indicative of a progressive increase in the reabsorption of a compound in the renal tubules   A ratio above 1 0 is indicative of tubular excretion, the extent of which is reflected in the value of the excretion ratio   Tubular excretion, in the case of this series of compounds, is presumably by an active process which specif ically extrudes the material into the lumen of the tubule (28)   The overall rate of excretion of a compound in the latter case is equal to the sum of its rate of filtration at the glomerulus and its rate of secretion by the tubules

A summary of the observations on the renal excretion of the compounds studied is given in tables 7 to 9   Each value is the mean of several experiments per formed on at least two dogs   Information is given on the creatinine clearance ratio which describes the overall rate of renal excretion, the fraction of the sulfon amide which is filterable from the plasma at a normal concentration of plasma albumin (c f 3), the pKa of the compound (c f 1), and its excretion ratio

The addition of a relatively non polar acyclic substituent to the sulfonamide complex may enhance or depress the excretion of the resulting compound to a variable extent (table 7)   Such changes in excretion are largely the result of a change in the extent to which the compound is reabsorbed, since this class of compounds does not appear to be bound extensively on plasma protein   How ever, when a strongly polar group with acidic properties is added, it may convert the compound into one which is actively secreted by the renal tubules as is the case with sulfanilylglycine and the N^1 acetyl derivative of sulfanilamide

It does not appear, however, that any generalization may be derived from these data since the results are not wholly consistent with the information ob tained on a series of simple compounds allied to sulfanilamide   It is apparent from the latter data that sulfanilic acid is reabsorbed to a slight extent and a similarly strong organic acid, paraminobenzoic acid, is reabsorbed to a consid erable extent   The excretion data on paraminobenzoic acid are particularly interesting in that it has a dissociation constant which is midway between that of sulfanilyl glycine and the N^1 acetyl derivative of sulfanilamide   However, paraminobenzoic acid is reabsorbed to an extent which suggests the intervention of an active tubular process whereas sulfanilyl glycine and the N^1 acetyl deriva tive of sulfanilamide are both actively excreted by the renal tubules

Other data in table 7 are of interest in that they indicate that metanilamide and orthanilamide are handled by the renal nephron in a manner which is quan titatively similar to that of sulfanilamide itself   This would indicate that the position of the substituent groups is of little importance in determining the ex cretion of the members of this family of three as it was of little consequence in determining their distribution in the body   It is of some interest to note that whereas the N^1 acetyl derivative is actively excreted, the N^4 acetyl derivative is more or less excluded from participating in renal tubular processes within the nephron (29)   It is also worthy of comment that even as the glycine derivative of sulfanilic acid is actively excreted by the renal tubules, so is the glycine deriva tive of paraminobenzoic acid, i e , paraminohippuric acid (30)

The data on the renal excretion of some of the isocyclic derivatives of sulfanil

amide are summarized in table 8. It may be noted that such compounds are, in general, bound extensively on the non-diffusible constituents of plasma; that the N^1 phenyl is itself actively secreted, whereas the $N^1$4-aminophenyl is reabsorbed to a considerable extent; that the sulfonic acid derivatives of N^1 phenyl

TABLE 7

Summary of observations on the excretion in the dog of a series of N^1-acyclic derivatives of sulfanilamide and a series of simple compounds closely allied to sulfanilamide

$$H_2N \underset{}{\bigcirc} SO_2NH-R$$
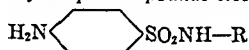
—R	pKa	SULFONAMIDE CREATININE CLEARANCE RATIO	PER CENT PLASMA SULFONAMIDE FILTERABLE	EXCRETION RATIO
—H	10.43	0.27	90	0.30
—CH₃	10.77	0.12	80	0.15
—CH₂CH₃	10.70	0.11	79	0.14
—OH		0.35	88	0.40
—NH₂	10.70	0.43	83	0.52
—CH₂CH₂OH	10.94	0.63	89	0.71
—C=NH·NH₂	*	0.76	94	0.81
—CH₂COOH	3.52	1.35	92	1.47
—COCH₃	5.38	1.19	87	1.37

Simple Allied Compounds

	pKa	SULFONAMIDE CREATININE CLEARANCE RATIO	PER CENT PLASMA SULFONAMIDE FILTERABLE	EXCRETION RATIO
$H_2N\bigcirc$ SO₂NH₂	10.12	0.17	73	0.23
$H_2N\bigcirc$ SO₂NH₂	10.12	0.25	89	0.28
OCH₂CHN$\bigcirc$SO₂NH₂		0.67	80	0.84
$H_2N\bigcirc$SO₂OH	3.20	0.89	98	0.91
$H_2N\bigcirc$COOH	4.68	0.18	93	0.19

* Very weak.

sulfanilamide are actively secreted as are the carboxy derivatives; but that although sulfonamide derivatives are actively secreted when the nuclear substituent is in the 3 or 4 position, this is not so with the substituent in the 2 position. There is also a suggestion that the position of the substituent group has some effect upon the rate of active transport in the group of sulfonic acid derivatives. The excretion ratio in this series increases progressively from 6 to 14,

as the nuclear substituent is rotated from the 4 to the 3 to the 2 position. The phenomenon is not apparent, however, in the case of the carboxy derivatives. It is not surprising to find that the position of a substituent may have an effect upon the extent to which a compound is bound on plasma protein or upon the rate of a biological process which involves a series of chemical combinations (28). It is somewhat surprising that the position can determine whether a substance is actively secreted or, as appears to be likely in the case for the sulfanilyl orthanilamide, actively reabsorbed.

The excretion data on some of the important heterocyclic derivatives of sulfanilamide are summarized in table 9. The series is small, but the data con-

TABLE 8

Summary of observations on the excretion in the dog of a series of N^1-isocyclic derivatives of sulfanilamide

$$H_2N \bigcirc SO_2NH \bigcirc \overset{2\ \ 3}{\underset{4}{}}$$
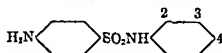
SUBSTITUENT	pKa	SULFONAMIDE CREATININE CLEARANCE RATIO	PER CENT PLASMA SULFONAMIDE FILTERABLE	EXCRETION RATIO
	9.60	1.00	28	3.99
4—NH₂	10.22	0.36	65	0.55
2—SO₂NH₂...		0.09	19	0.56
3—SO₂NH₂	8.23	0.73	38	1.92
4—SO₂NH₂	7.85	1.34	56	2.39
2—COOH	3.85	0.77	12	6.42
3—COOH ..	4.10	1.84	15	12.26
4—COOH....	4.05	1.67	34	4.91
2—SO₂OH	3.40	2 22	16	13.90
3—SO₂OH	3.35	2 30	24	9.58
4—SO₂OH . ..	3.40	2.23	34	6.29

tain several points of considerable interest. They indicate that sulfapyridine, sulfadiazine and sulfamerazine are reabsorbed to a considerable extent in the renal tubules, whereas sulfathiazole and sulfamethylthiazole appear to be excreted by a process which is almost exclusively that of glomerular filtration. The latter conclusion follows from the values of the excretion ratios given in the last column of the table which, in the case of the thiazoles, approximate a value of 1.0.

Reabsorption is considerable in the case of sulfamerazine, i.e., 4-methyl sulfadiazine and, as in the case of paraminobenzoic acid, is in the order of magnitude which suggests that reabsorption proceeds as an active tubular process. When it is appreciated that such a diffusible substance as urea is only reabsorbed to the extent of some 40 per cent, at ordinary urine flows, then it would be surprising
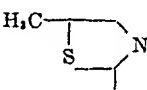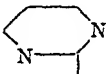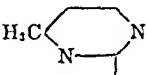
if organic complexes of the size and shape of sulfamerazine and paraminobenzoic acid are reabsorbed to the extent indicated without the intervention of an active process.

DISCUSSION. The study as a whole should not be taken as a definitive description of the relationship between the chemical structure of these compounds and their physiological disposition. It is at best a survey indicating the worthwhileness of this approach to the problem.

TABLE 9

Summary of observations on the excretion in the dog of a series of N^1-heterocyclic derivatives of sulfanilamide

$$H_2N\bigcirc SO_2NH-R$$

—R	pKa	SULFONAMIDE CREATININE CLEARANCE RATIO	PER CENT PLASMA SULFONAMIDE FILTERABLE	EXCRETION RATIO
(pyridine ring)	8.44	0.38	69	0.55
(thiazole ring)	7.12	0.40	40	1.00
(methylthiazole ring)	7.80	0.25	23	1.09
(pyrimidine ring)	6.48	0.27	83	0.33
(methylpyrimidine ring)	7.06	0.13	61	0.21

It has been demonstrated that a change in chemical structure within this small series of compounds is accompanied by drastic consequences to those factors which together determine the physiological disposition of a compound. Were one to assume some common chemotherapeutic effect on the part of all compounds of the series, it is doubtful whether sulfanilic acid or compounds comparable to it from the standpoint of distribution would be effective if the site of action was within the central nervous system, unless there was some change in the characteristics of the blood-brain barrier. This follows from the fact that compounds of the latter type are excluded from this compartment of the body by the normal blood-brain barrier. Similarly, one could reason that if it were desirable to obtain a temporary high concentration of one of these compounds

in the extracellular fluid compartment in order to accomplish a given end, then the compound of choice would have properties similar to sulfanilic acid, or sulfanilyl sulfanilic acid. This follows from the limitation imposed on the distribution of the compounds together with their relatively high excretion rates the latter largely determining the duration of the desired effect. For longer action, one would select a compound with the lower excretion rate. If one were not concerned with a limitation on the distribution of a compound and wished an effect with considerable duration then one would select a compound such as sulfanilamide which has a large volume of distribution together with a low excretion rate or a compound such as sulfamerazine specifically because of the low excretion rate.

In other words, there is some justification for the belief that it may ultimately be possible to limit both the theatre of operation of an active substance within a series as well as the duration of the effect produced through the proper modification of chemical structure. The data on the series of sulfonamides examined do not lead one to suppose that this will be a simple procedure. Wholly apart from the effect of a chemical change in fundamental activity of the resulting compound, at least two factors will be concerned with the distribution and excretion. The strength of acidic groups is one of these, whereas the other apparently relates to the molecular structure of the compound as a whole. The latter factor is difficult to define in terms of measurable entities at the moment, but is none the less important.

The results on the distribution of the compounds in the body are fairly consistent in that the ability of a compound to pass a cell membrane appears to be governed in no small measure by the extent of its dissociation at blood pH. Those compounds with a high pKa are permitted to enter the tissue cells quite freely, whereas those with a low pKa have in general a more restricted distribution. In this respect, then, sulfonamides with some exceptions follow the accepted concepts of permeability of cell membranes to organic electrolytes (36).

The observations on cerebrospinal fluid offer additional evidence that the normal barrier between the blood and cerebrospinal fluid is a highly selective one, and that the mechanism of formation of this fluid is not one of simple filtration. It has been shown previously in studies on the distribution of thiocyanate, bromide and iodide, that cerebrospinal fluid is formed not only at the choroid plexus but also by the passage of fluid and solute from blood into the extracellular fluid of the brain and then into the subarachnoid space (19 20). The data are wholly consistent with the view that normal brain tissue must be considered to be in equilibrium with a fluid having the characteristics of cerebrospinal fluid rather than the characteristics of plasma water. The behavior of the normal blood brain barrier is quite different from that of the ordinary cell membranes in that the molecular configuration of a substance, apart from its acidic properties, seems to be important in determining the ability of a substance to gain entrance into cerebrospinal fluid and the interstitial fluid of brain substance. The brain capillaries and their surrounding pia do not offer a satisfactory anatomical explanation of such selectivity.

The relation between the chemical structure of organic compounds and their ability to participate in the active excretory mechanisms of the renal tubule has been the subject of previous investigations by others.   A large number of dyes as well as several sulfanilamide derivatives have been examined, using the perfused frog kidney as an indicator.   It was concluded from these studies that the polarity of the molecule largely determined whether a compound participated or did not participate in a mechanism of active tubular excretion.   Those compounds with polar-non-polar or socalled organophilic-hydrophilic configuration appeared to be secreted, while non-polar molecules did not (31, 32, 33, 34, 35). Many of the actively secreted compounds in the present series possess such a polar-non-polar configuration, but there are several striking exceptions.   For example, sulfanilyl sulfanilamide is actively secreted, yet it contains two weakly polar groups, an amino and a sulfanilamido group.   Sulfanilic acid and paraminobenzoic acid are strongly polar molecules and are not secreted, whereas comparable compounds such as the sulfanilyl glycine, the N^1-acetyl sulfanilamide and paraminohippuric acid are.   Molecular configuration is undoubtedly important in determining the participation or non-participation of a compound in a process of active tubular excretion.   However, the mechanisms which are involved require further clarification.

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INDEX